Research Article

Uncanny Segmental Molecular Integration of Ahsv and Eev with Btv in Sheep and Goats in Egypt

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Abstract | This study was conducted principally as a trial to expose the molecular identity of the encountered BTV virus and consequently justifying the PCR negativity encountered before. Therefore, the proteomic approach was utilized. The virus was isolated on SPF-ECE and concentrated using PEG-6000. The concentrate was analyzed using liquid chromatography with tandem mass spectrometry (LC-MS/MS) using a technique called untargeted (label-free) shotgun proteomic analysis. The resultant peptides were used along with RefSeq proteins and primers for multiple sequence alignment. Multiple extrinsic proteins [NS1, NS3 and VP3 of African Horse Sickness Virus (AHSV) and NS3 of Equine Encephalosis Virus (EEV)] were detected and at the same time, integration events were examined in this study. This research work entails unpredicted integration of foreign non-BTV viral proteins (NS1, NS3 and VP3 of AHSV and NS3 for EEV). The multiple sequence alignment models elucidated the extent of the integration into the BTV genomic backbone with a resultant insight into PCR negativity. The result confirms the unexpected integration of some segments from equine viruses AHSV and EEV within BTV genome.

Keywords | African Horse Sickness Virus, Equine Encephalosis Virus, shotgun proteomics, Bluetongue Virus, Egypt.

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INTRODUCTION

Bluetongue virus in Egypt

The Bluetongue virus (BTV), in the *Reoviridae* family (genus *Orbivirus*), causes bluetongue disease in livestock across the world and is a vector-borne disease (VBD) of significant economic importance (El Moustaid et al., 2021). The virion is an icosahedral particle assembled as a triple-layered capsid with viral genomes composed of 10 segments (Seg-1 to Seg-10) of double-stranded RNA (dsRNA) encoding seven structural proteins (VP1 to VP7) and five non-structural proteins (NS1, NS2, NS3/NS3a, NS4 and NS5). Currently, BTV is classified into 27 antigenically distinct, which suggests an ongoing emergence of atypical serotypes of BTV (Yang et al., 2021). BTV was first described in South Africa in 1876 when European sheep intensive farming was introduced in the region (Alkhamis et al., 2020), but in Egypt, its first referral was as early as 1965 (Hilmy Zaki, 1965) while identification of BTV association with epizootics was at 1970 and 1972 (Ayoub and Singh, 1970; Soliman et al., 1972) with discovery of serotypes 3, 4, 13, 15 and 16 at different time intervals (Hafez and Ozawa, 1981; Ahmed et al., 2019;

OIE, 2019).

African horse sickness virus in Egypt

The African horse sickness (AHS) is an infectious, non-contagious, notifiable disease with high fatalities among susceptible hosts and particularly horses with potential rapid universal spread. The virus is another member of genus *Orbivirus*, family *Reoviridae* with potential rapid universal spread (Dennis et al., 2019). The first reported outbreak of the disease was in 1928 followed by 1943 then 1953 and 1971 and all were in equines in Upper Egypt then three AHS viruses (type 9) were identified in street dogs (Salama et al., 1981). According to OIE, there was an official notification in Egypt in year 1959 in equine then in 1994; there was suspension of vaccination followed by declaring the country provisionally free since 1996 and only equids are believed to play important role in maintaining or amplifying the disease (OIE, 2020).

Equine encephalosis virus in Egypt

The Equine encephalosis is a febrile, mild, transient, non-contagious and rarely fatal disease of horses. It was first isolated in year 1967 in South Africa from a thoroughbred mare named Cascara and died following febrile nervous disease. The virus is another member of genus *Orbivirus*, family *Reoviridae*. Seropositivity for the virus has been described in donkeys and zebras in some African countries. Egypt's bordering countries such as Israel, Palestinian territories and Jordan have encountered serological evidences and it was isolated during an outbreak of febrile horses in Israel (Tirosh-Levy and Steinman, 2022).

Shotgun proteomics and Bluetongue virus

The full integrative study of proteins and their biological activities is known as proteomics. The goal of proteomics, as in our study, is to produce a complete map of a species' proteome. The bottom-up proteomic approach is often obtained using a combination of liquid chromatography (LC) and tandem mass spectrometry (MS/MS) (Zhang et al., 2013; Nesvizhskii, 2014). At present, the usage of mass spectrometry-based techniques has become a core principal in viral particle identification (Milewska et al., 2020). LC/MS-MS usage in Bluetongue virus identification was not previously documented worldwide. The same technique was utilized in two studies, although in quantitative form, to find differentially expressed proteins in BTV-infected sheep testicular cells (Du et al., 2016) and to identify intracellular signaling pathways and novel host-cell kinases involved during BTV infection (Mohl et al., 2017).

The objective

During the team's laboratory studies on Bluetongue virus (El-Sebelgy et al., 2021), numerous attempts to amplify and molecularly characterize the virus using several highly conserved genes-based primers (VP3, NS1 and NS3) were met with failure and frustration as all clinical, serological and virological studies were pointing strongly to the identity of the virus as Bluetongue. Therefore, this study was conducted principally as a trial to expose the molecular identity of the virus via shotgun proteomics as an alternative method to RT-PCR.

MATERIALS AND METHODS

Virus Isolation

The buffy coat isolated from serologically positive clinically infected Baladi sheep and goats, initially screened by IDEXX c-ELISA, was used for isolation via the yolk sac route/CAM route as primary isolation. 0.3 ml of buffy coat (inoculum) was injected aseptically intrayolk in 10-day-old SPF-ECE or 13-day-old via CAM, sealed, incubated for 3-5 days, eggs dying within 24 hours were discarded, tissues (brain, liver, spleen, heart, and lungs) from the whole embryo for yolk sac route or CAM in case of CAM route and both removed aseptically, emulsified and tissue homogenate was stored in the test tube at -20°C till usage for subsequent passages. A total of 12 passages were carried out (Clavijo et al., 2000).

Virus concentration and partial purification

PEG-6000 (Sigma-Aldrich) along with high-speed centrifugation was used to obtain virus concentrate. Chicken embryo tissue homogenate undergone several filtration steps (filter papers and 0.45 µm syringe filter). Sodium chloride to a final concentration of 2.3% and PEG-6000 to a final concentration of 7.0% were added to the filtrate. After several steps of cooling and centrifugation, PEG was removed by pelleting and the virus-rich supernatant in isotonic TES (Tris HCl-EDTA-NaCl) was collected in Eppendorf tubes, sealed with parafilm tape, and stored at -20°C (Alves, 2015).

Preparation of sample for analysis of the viral proteome

According to Enany et al. (2020), Magdeldin et al. (2014), Saadeldin et al. (2020) and Wisniewski,Gaugaz (2015), the order and details of sample preparation, chromatography, mass spectrometry, data processing and analysis were accomplished.

Protein extraction and denaturation

The sample was provided as virus concentrate 200 µl aliquots in TES buffer. Five samples were pooled to be 1 ml and 1 ml 8M urea (500 mM Tris pH 8.5) was added on each sample. Acetone was added on the sample (3x times); sample was incubated at 80 °C for 30 minutes, then at -20 °C overnight and centrifuged at 10,000 rpm for 30 minutes. The supernatant was discarded and the tube was left

at room temperature (RT) to all evaporation of the rest of the acetone. After that, 30 µl 8M urea was added on the extracted proteins and the sample was shaken vigorously and centrifuged at 10,000 rpm for 30 minutes at 4 °C.

Protein digestion

The sample was digested in three steps; reduction, alkylation and trypsinization. For the first step, reduction was commenced by adding 2 µl of 200 mM dithiothreitol (DTT), vortexed, spun down then incubated for 45 minutes at RT. For the second step which is alkylation, 2 µl of 1M iodoacetamide (IAA) were added, incubated at RT for 45 minutes in the dark and 102 µl of 100 mM Tris pH 8.5 were supplemented. For the last step in this phase and which is trypsinization, 6 µl trypsin (containing 1 µg procaine enzyme) were added, incubated overnight at 37 °C with shaking at 900 rpm then 6 µl of 100% formic acid (to acidify the sample to pH 2-3) were added and finally spun down for 30 minutes at RT.

Stage tip (MonoSpin Reversed Phase Columns) prod#5010-21701

50 µl of methanol on the tip were added for activation, followed by 50 µl from solution B comprising from 0.2% formic acid (FA) + 80% acetonitrile (ACN) for initialization then 50 µl from solution A (0.2% FA) twice for re-equilibration. For sample trapping, the Eppendorf tube was changed and the sample added followed by washing using 15 µl solution A twice and finally for elution, collection tube was recovered 3 times with 30 µl solution B, speed-vaced then reconstituted in 20 µl solution A and the sample was subjected to peptide quantification. In this phase of the experiment, centrifugation at 3,000 rpm was done between each step in Stage tip.

Chromatography

The liquid chromatography system used was NanoLC system consisting of Eksigent nanoLC 400 autosampler attached with Ekspert™ nanoLC425 pump with injection volume of 1 µg/10 µl, injection mechanism as trap and elute, needle was for two cycles using 10% isopropanol for 55 minutes as analysis time. The sample was cleaned up using trapping cartridge ChromXP C18CL 5µm (10 x 0.5 mm) pumped at flow rate of 10 µl/minute for 3 minutes using mobile phase A (MilliQ containing 0.1% FA **(A)** and acetonitrile containing 0.1% FA **(B)**.

Gradient Profile

Mass Spectrometry

The LC-QTOF system used was SCIEX TripleTOF™ 5600⁺ with acquisition mode as positive, TOF mass range as 400-1250 m/z, MS2 range (product ion) 170-1500 m/z, ion selection threshold set as 150 cps and total run time as 55 minutes. High resolution TOF MS survey scan was followed by product ion scan for the most abundant 40 ions with cycle time as 1.5 second and MS calibration [SCIEX tuning solution (p/N 4457953)].

The gradient profile used during the course of the chromatographic run is mentioned in the following Table (1)

Table 1: The gradient profile used during the course of the chromatographic run

Time (min)	A%	B%
0	97	3
38	70	30
43	60	40
45	20	80
48	20	80
49	97	3
59	97	3

Data Processing

PeakView® 1.2.0.3. was used for the qualitative review of LC/MS data in Analyst® TF 1.7.1. software (used for data acquisition), while raw MS files from the TripleTOF® 5600⁺ were analyzed by ProteinPilot™ (version 5.0.1.0., 4895), Paragon™ algorithm (version 5.0.1.0., 4874). Bluetongue virus (Swiss-Prot and TrEMBL containing 6,047 entries) was used as the database with search parameters as following; cysteine alkylation using iodoacetamide, digestion with trypsin, denaturation buffer containing urea as a special factor, thorough search effort, biological modifications as ID focus and false discovery rate (FDR) analysis and bias correction set to yes.

Bioinformatics analysis

All the peptides either from BTV, AHSV or EEV were reversely translated to its most probable genomic origins using EMBOSS (The European Molecular Biology Open Software Suite) Backtranseq (Madeira et al., 2019). The alignment of all aforementioned with BTV NS1, NS3 and VP3 reference sequences, along with primers, was done using Biological Sequence Alignment Editor, v 7.2.5 (BioEdit software) with ClustalW embedded application (Hall, 1999) and visualized, examined and saved using SnapGene® software version 6.0.4 (from Insightful Science; available at snapgene.com). All BTV RefSeq and primers used in the analysis are listed in Tables (2) and (3).

Figure 1: Showing AHSV VP3 peptide 1 integration with BTV VP3

Table 2: BTV primers used for amplification trials

Primer	Sequence	Position	T_m	Product Size (bp)	Target Segment (Protein)	Reference
BTVs3gp1-F	CTGCTCCCATCACTACAACCA	Forward	60	312	Seg-3 (VP3)	(Kim et al., 2017)
BTVs3gp1-R	AAACCTCACCCAGTCGTCATC	Reverse	60	312	Seg-3 (VP3)	(Kim et al., 2017)
BTVs5gp1-F	CATCATTCGTGAGGGGTTGGA	Forward	60	370	Seg-5 (NS1)	(Kim et al., 2017)
BTVs5gp1-R	CTCCTGCCATCCTTCATCGTT	Reverse	60	370	Seg-5 (NS1)	(Kim et al., 2017)
BTVs10gp1-F	TCGCTGCCATGCTATCCG	Forward	56	251	Seg-10 (NS3)	(Akita et al., 1992)
BTVs10gp1-R	CGTACGATGCGAATGCAG	Reverse	56	251	Seg-10 (NS3)	(Akita et al., 1992)

Table 3: BTV reference sequences

BTVprotein/segment	NCBI reference sequence accession number
VP3/segment 3	NC_006014.1
NS1/segment 5	NC_006025.1
NS3/segment 10	NC_006015.1

Figure 2: Showing BTV NS1 and forward primer mismatching

Figure 3: Showing BTV NS1 and reverse primer mismatching

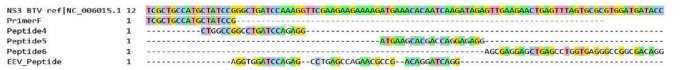

Figure 4: showing EEV NS3 integration with BTV NS3 and forward primer mismatching

Figure 5: showing AHSV NS3 peptide 3 integration with BTV NS3 and reverse primer mismatching

Figure 6: showing AHSV NS3 peptide 2 integration with BTV NS3

Table (4) is representing non-BTV distinct peptide summary including protein names, peptide sequences, post-translational modifications, cleavages, observed molecular weight and sum MS2 counts. The mode of transmission and hosts are not included originally in the distinct peptide summary generated by ProteinPilot™ and are included as additional information and for illumination and confirmation that both viruses (AHSV and EEV) mainly affects equines and both are transmitted by Culicoides species same as Bluetongue virus. Sum MS2 counts represents roughly the amount of that peptide in the total mix of peptides obtained. Tables (5), (6) and (7) are listing in order peptides related to VP3, NS1 and NS3 in addition to modifications, cleavages, observed molecular weight and sum MS2 counts. Tables (4), (5), (6) and (7) were used along with Table (2) for primers and Table (3) for BTV reference sequences for multiple sequence alignment model to clarify how primers are met with mismatches and how non-BTV peptides are incorporated within Bluetongue virus segments VP3, NS1 and NS3.

The figures discussed below are showing only the regions of multiple sequence alignments, but the full-length alignment of the discussed BTV proteins are shown in the supplementary materials. In Figure (1), 8 similarities are shown between VP3 peptide 1 and AHSV peptide 1 and 10 mismatches including 2 gaps at residues 1013 and 1014 and so along the length of AHSV peptide of 30 residues, about 25% of it is mismatches. Furthermore, the absence of primers nucleotides mismatching in this pair and absence of BTV segments in primers-binding sites could be indicative of presence of other foreign molecular fragments in those sites, especially with negativity of PCR using that pair. In Figure (2), the forward primer along with RefSeq of BTV NS1 is completely matching as expected, while several mismatches (3) are shown with BTV peptide 3 and peptide 9 has no mismatches except for a gap of 4 residues at position 974-977 and so the gap along with 3 mismatches, accounting for about 30% of primer's length, that could clarify the failure of PCR due to the crucial primer-binding sites difference. As for the reverse primer shown in Figure (3), partial matching consisting of 3 residues, which even if theoretically participated in a PCR reaction, will not achieve exponential amplification and a good yield, as both primers are needed to achieve

Table 4: African Horse Sickness Virus and Equine Encephalosis Virus discovered peptides as non-BTV distinct peptide summary

Protein Names	Mode of transmission	Hosts	Peptide Sequences	Modifications	Cleavages	Observed Molecular Weight	Sum MS2 Counts
African Horse Sickness NS1	Insect-borne, transmitted by *Culicoides* species (Moss et al., 1992)	Mainly equids and zebras act as reservoir host (Gao et al., 2022)	QRNRPSR		missed R-N@2	912.50	3,466.02
			LTCFAIF GCKPTAR	Carbami-domethyl(C)@3; Carbami-domethyl(C)@9		1,640.87	1,773.69
			GERANAVR		missed R-A@3	871.40	324.49
African Horse Sickness NS3			TELTTMR	Carbamyl(R)@7		893.42	3,448.06
			RDIAKR	Carbamyl(K)@5	missed R-D@1; missed K-R@5	800.44	1,695.89
			DEKAAFGA-MAEALR	Carbamyl(K)@3	missed K-A@3	1,521.79	426.15
African Horse Sickness VP3			VINAPPNSFR			1,113.58	2,221.40
Equine Enceph-alosis virus NS3		Equid species including horses, donkeys and zebras (Tirosh-Levy and Steinman, 2022)	RWIQSLSQ NADRIR		missed R-W@1; missed R-I@12	1,741.88	734.77

Table 5: Distinct peptide summary of BTV inner core protein (VP3)

Peptide Sequences	Modifications	Cleavages	Observed Molecular Weight	Sum MS2 Counts
KRITYSR		missed K-R@1; missed R-I@2	922.49	5,233.39
KPNISFRHIVMQSR		missed R-H@7	1,711.89	2,824.49
DMLPEPR			856.43	2,416.71
EADFTVPNVQK	Carbamyl(K)@11		1,289.55	1,384.77
HLSLMQRR		missed R-R@7	1,039.47	1,278.72
MAAQNDQR	Deamidated(N)@5		933.44	5,296.43
GKGSFMLHDIPTR			1,457.65	1,110.70
VAQNAQATER	Deamidated(N)@4		1,087.55	4,169.89
AVLNDSSEAVK			1,131.58	2,785.11

Table 6: Distinct peptide summary of BTV non-structural protein 1 (NS1)

Peptide Sequences	Modifications	Cleavages	Observed Molecular Weight	Sum MS2 Counts
AMIAAADAEEPAK	Carbamyl@N-term		1,329.68	7,912.23
DRSIVEAR			944.48	1,186.87
EGLACAEHWEWLL-DYMNGK	Carbamidomethyl(C)@5; Deamidated(N)@17		2,322.09	14,371.64
EGVFMLGRVLLR	Oxidation(M)@5	missed R-V@8	1,404.72	3,280.75

GEHNQIKR		missed K-R@7	980.55	2,638.87
GQHNQIQR			979.53	1,485.87
KCGALMLENYR	Carbamidomethyl(C)@2	missed K-C@1	1,353.73	1,271.54
LADITGCSPFREVIIR	Carbamyl@N-term; Carbamidomethyl(C)@7	missed R-E@11	1,889.00	5,344.29
RSGNGNWQSWLLPMTIVR	Carbamyl@N-term	missed R-S@1	2,157.06	5,433.42
VFQVMFLP		cleaved P-I@C-term	979.47	2,163.38

Table 7: Distinct peptide summary of BTV non-structural protein 3 (NS3)

Peptide Sequences	Modifications	Cleavages	Observed Molecular Weight	Sum MS2 Counts
AEKAAFASYR	Carbamyl@N-term		1,155.57	3,069.71
VMKKPSYYDAVR			1,584.86	4,048.83
FVNLSLVR	Deamidated(N)@3		947.45	10,606.59
LAGLIQR	Carbamyl@N-term	cleaved M-L@N-term	812.41	1,117.35
MKHDQER			942.34	926.87
SEELSLVRAGDR			1,330.63	3,900.79
SERGLNQHIDMIR		missed R-G@3	1,567.78	1,798.60

this outcome. In Figure (4), the forward primer along with RefSeq of NS3 is completely matching as expected, while 2 mismatches are shown with BTV peptide 4 and one with EEV peptide, while EEV peptide has 9 mismatches with peptide 4 and 5 and gaps at residues 43-44 and 62-63 with peptide 6 starting and continuing just after peptide 5 forming a continuum with peptide 5. Furthermore, in Figure (5), the reverse primer has relatively large number of mismatches (7) accounting for about 40% of its length and so both forward and reverse primers (Figure 4 and 5) have mismatches. As for AHSV peptide 3, several mismatches (8) are also encountered with one gap at residue 263 in relation to peptide 1. As for Figure (6), AHSV peptide 2 has 6 mismatches when compared to peptide 7, while peptide 7 is forming a continuum when coupled with AHSV peptide and peptide 2. As shown in Figures (2), (4) and (5) primers' mismatching is self-explanatory for RT-PCR failure.

As shown in Figures (1), (5) and (6), AHSV peptides had the greatest share in integration with BTV and this can be attributed to morphological (almost identical) and biochemical resemblances that is indicative of similar means of replication and assembly of the two viruses (Dennis et al., 2019).

It is worth mentioning that the presence of these foreign peptides related to AHSV and EEV is unusual and very awkward to be found within BTV genome, especially with the fact that both viruses are equine viruses, and not of small ruminants as sheep and goats, where the virus was isolated from. Additionally, according to OIE, Egypt was declared free of AHS disease at 1996 and there were no official notifications from 2005 till now and EEV was never officially reported in the Egyptian field.

CONCLUSION

The existence of foreign equine viral fragments of AHSV and EEV integrated within BTV genome from virus isolated from sheep and goats in the Egyptian field. So, the occurrence of those integrative parts warrant comprehensive and in-depth investigation to find how these mutations can modulate the Bluetongue virus isolated, especially regarding its infectivity, pathogenicity and behavior.

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AUTHORS CONTRIBUTIONS

MMS and KSZ collected field specimens and isolated the virus. HMM and SMT carried out conceptualization of the research article. All authors shared in reading, critically analyze and approval of the final manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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