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ORIGINAL ARTICLE

Studies on Bluetongue Virus (BTV) Isolated from Clinical Field Specimens of Ruminants during 2016 in Egypt

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Abstract
Bluetongue disease (BT) is a notifiable disease affecting ruminants causing devastating economic losses globally. In the current study, the prevalence of Bluetongue virus (BTV) antibodies in sera of clinically infected Baladi and Merino sheep, goats, and cattle, from South Sinai areas and Monib abattoir (during Eid El-Adha), were investigated by competitive ELISA. Seropositivity of goats and Baladi sheep sera were 77.8% and 46.3% denoting to the highest prevalence of BT virus in these animals, but sera of Merino sheep and cattle were negative denoting to the freedom of this lot of imported animals from previous vaccination or infection. BTV was isolated on SPF-ECE via yolk sac route yielding hemorrhagic embryos, also on (CAM) with the appearance of pock lesion, but secondary isolation on (BHK-21) demonstrated cytopathic effects (CPE) typical of viral infection. Concentration of BTV was performed using PEG-6000. BTV and aggregation of virus particles were observed with specific BTV antibodies and photographed by electron microscopy (EM). Titration studies (TCID50) was approximately 10-7 TCID50/ml. Finally, the identification of Bluetongue virus was accomplished and confirmed molecularly using shotgun proteomic analysis demonstrating the presence of VP1 protein during year 2016 in Egypt.

Keywords
Bluetongue virus (BTV), Egypt, Electron Microscopy, ELISA

1. Introduction
Bluetongue disease (BT) is a notifiable arboviral disease affecting ruminants causing severe economic losses worldwide. The BTV can infect nearly all ruminants and is largely transmitted via biting midge (*Culicoides spp.*) among other arthropods. Bluetongue disease virus (BTV) is a non-enveloped, spherical double-stranded RNA virus, with 3 concentric protein shells, belonging to genus *Orbivirus* and family *Reoviridae*. The RNA codes for 5 non-structural proteins (NS1, NS2, NS3/NS3A, NS4 and NS5) and 7 structural proteins (VP1–VP7) **(Stewart et al., 2015; Pullinger et al., 2016)**. There are 27 antigenically distinct BTV serotypes identified among several other tentative serotypes **(Bitew et al., 2013)**.

Several types of ELISA test kits are available worldwide for the detection of BTV antibodies. Common ELISA antibody detection methods include iELISA (indirect ELISA) **(Tigga et al., 2015)** and cELISA (competitive ELISA) **(Zaher and Ahmed, 2015a; Hassine et al., 2017; Bakhshesh et al., 2020)** and the latter being the most popular and is considered the prescribed test for international trade by OIE **(OIE, 2018)**.

Primary isolation of BTV is usually performed on ECE or KC cells followed by BSR cells **(Verdezoto et al., 2018)**, directly on BHK-21 **(Chauhan et al., 2014)**, ECE **(Balaro et al., 2014)**, KC cells **(Veronesi et al., 2013)** or KC cells followed by Vero cells **(Lorusso et al., 2018)**. Secondary isolation of BTV on cells, either mammalian such as BHK-21 or Vero cells; or insect cell cultures such as KC cell and C6-36 is a well-documented and accepted practice **(Mertens *et al.*, 2009; Krishnajyothi et al., 2016)**. Another uncommon/rarely used route of primary isolation and inoculation is CAM **(Cherrington et al., 1985b)**.

The process of BTV concentration helps in its visualization using EM. Among different methods used is high-molecular-weight PEG-6000 macromolecular proteins, pioneered by **Yamamoto et al. (1970)** for bacteriophages **(Killington et al., 1996; Alves, 2015)**. Electron microscopy and/or immune-electron microscopy are considered the most powerful and robust tools for meticulous biomolecule structure analysis and observation at a near-atomic level **(Murata and Wolf, 2018; Rojas et al., 2019)**. $TCID_{50}$ and neutralization tests are very popular in the field of diagnostic virology and are used for titration and antigen detection **(Hierholzer and Killington, 1996; Madbouly et al., 2005)**.

The present study was designed to investigate BTV seroprevalence among ruminants in Egypt in the year 2016 along with virological studies (isolation, identification, and titration) to identify and characterize the virus.

2. Materials and Methods

2.1. Blood samples

A total of 57 pooled blood samples (originally 285) were aseptically collected in vacutainer tubes; both plain and EDTA. The plain ones were used to separate sera and EDTA-coated tubes were used for fresh whole blood to separate buffy coat using Lymphosep® (Lymphocyte separation medium – Biowest® - Cat. no. L0560-100) to be used later for virus isolation.

2.2. Serological assay

Pooled serum samples were examined using a commercially available c-ELISA kit (Bluetongue virus (BTV) VP7 Antibody Test Kit, IDEXX Bluetongue Competition, REF P00450-5) to screen for BTV-specific IgG antibodies following the manufacturer's instructions. The samples were considered positive when the optical density is less than or equal to 70% of the negative controls mean.

2.3. Isolation and concentration of BTV

2.3.1. Primary virus isolation using yolk sac route

The buffy coat isolated from serologically positive Baladi sheep and goats were used for isolation via the yolk sac route using 10-day-old SPF-ECE **(Clavijo et al., 2000)**.

2.3.2. Primary virus isolation using CAM route

A select of 0.1 ml of inoculum (tissue homogenate from SPF-ECE injected via yolk sac route) was injected in 13-day-old SPF-ECE via CAM **(Gray and Bannister, 1961; Cherrington et al., 1985b)**.

2.3.3. BTV concentration and partial purification

PEG-6000 (Sigma-Aldrich) along with high-speed centrifugation was used to obtain virus concentrate starting initially with chicken embryo tissue homogenate **(Killington et al., 1996)**.

2.3.4. Secondary virus isolation using BHK-21 cell line

Tissue filtrate and virus concentrate were utilized for secondary virus isolation on BHK-21 cell line following instructions by **American Type Culture Collection (ATCC) (2020)**.

2.4. BTV titration (TCID50 and SNT)

$TCID_{50}$ ((Median Tissue Culture Infectious Dose) was performed according to **Hierholzer and Killington (1996)** employing BHK-21 cell line. Serum Neutralization Test (SNT) was accomplished according to **Hsiung et al. (1994)** and **Madbouly et**

al. (2005) with the use of control positive BTV antiserum (GD Diagnostics – Cat. No. VLDIA260).

2.5. BTV electron microscopy

2.5.1. Virus concentrate

The virus-concentrated samples were pooled into one sample suspended in TES buffer then one drop of this suspension was employed for negative staining technique using phosphotungstic acid at Central Unit for Analysis and Scientific Services – NRC - TEM Unit using model JEM-2100 (JEOL, Japan) **(Zaher, 2012)**.

2.5.2. Pock lesion on CAM

The CAM sample with pock lesions was processed and stained for negative staining technique using uranyl acetate and lead citrate and then examined using TEM in Cairo University Research Park - Faculty of Agriculture model JEM-1400 (JEOL, Japan) **(Bozzola, 2014)**.

2.6. BTV immunoelectron microscopy

Equal volumes of virus and antiserum were mixed, prepared and stained with phosphotungstic acid for negative staining procedure followed by examination at Central Unit for Analysis and Scientific Services – NRC - TEM Unit using model JEM-2100 (JEOL, Japan) **(Chrystie, 1996)**.

2.7. Shotgun proteomic analysis

The virus concentrate obtained was analyzed using liquid chromatography with tandem mass spectrometry (LC-MS/MS) using untargeted (label-free) shotgun proteomic analysis and it was performed at Children's Cancer Hospital – Basic Research Department – Proteomics and Metabolomics Research Program.

3. Results

Blood sampling

The number, type, locality, and distribution of examined and sampled animals are summarized in **Fig. 1 of Bar chart** while **Fig. 2** showing clinically affected goats.

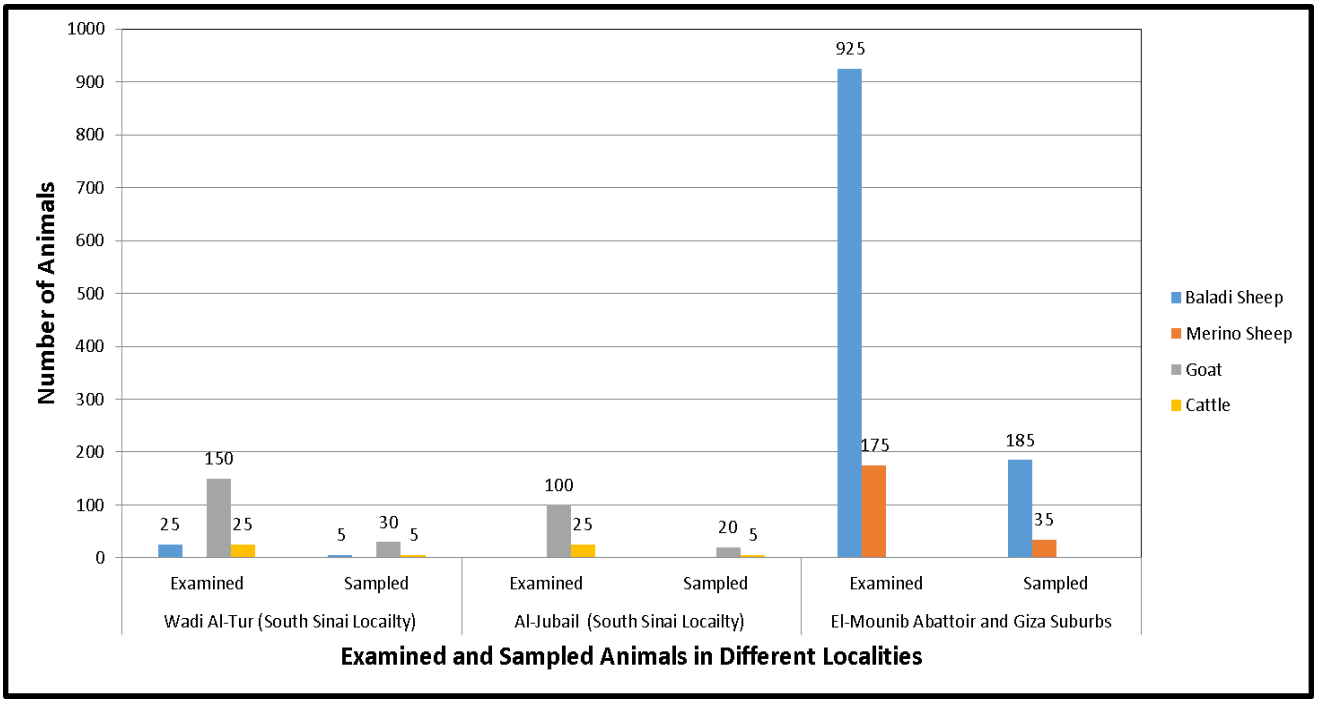

Fig. 1. Bar Chart showing examined and sampled animals (Baladi, Merino Sheep, Goat, and Cattle) among different localities

Table (1): BTV VP1 (RNA-directed RNA polymerase) peptides

Peptide Sequences	Modifications	Cleavages	Observed Molecular Weight	Sum MS2 Counts
EAVAPYR			804.40	6,911.84
RGIMSTTK		missed R-G@1	892.43	4,436.08
SKGTFKIPK		missed K-G@2; missed K-I@6	1,004.52	2,988.65
IAVTDDAKKR	Carbamyl@N-term; Deamidated(R)@10	missed K-K@8; missed K-R@9	1,159.50	2,600.71
YEGYTLEQIIEFGYGEGR	Carbamyl@N-term		2,165.99	2,455.28
SIGGDDRNSK	Carbamyl(K)@10	missed R-N@7	1,090.58	2,195.93
KDVVMRGFITANTILSVIEK		missed K-D@1; missed R-G@6	2,233.17	2,090.36
AKSEMQIFGDVPIK	Deamidated(Q)@6	missed K-S@2	1,562.83	2,081.68
AITVQGAQLIK	Deamidated(Q)@5	cleaved V-A@N-term	1,141.69	2,025.58
VAITVQGAR		cleaved M-V@N-term	913.49	1,864.59
IQKMEIGPR	Carbamyl(R)@9	cleaved Y-I@N-term; missed K-M@3	1,113.62	1,712.19
MRIVVTDDAK	Carbamyl(K)@10	missed R-I@2	1,189.68	1,577.50
HGTKYLR		missed K-Y@4	873.41	1,414.34
SQVQTMTTKVSR	Oxidation(M)@6	missed K-V@9	1,380.63	1,402.65
IAVTDGAKKR		missed K-K@8; missed K-R@9	1,057.56	1,086.74
TVQGAQLIKR		cleaved I-T@N-term; missed K-R@9	1,112.63	730.94
IIDFGYGEGR		cleaved Q-I@N-term	1,125.58	434.46
FSDHIRRIR		missed R-R@6; missed R-I@7	1,198.69	377.67

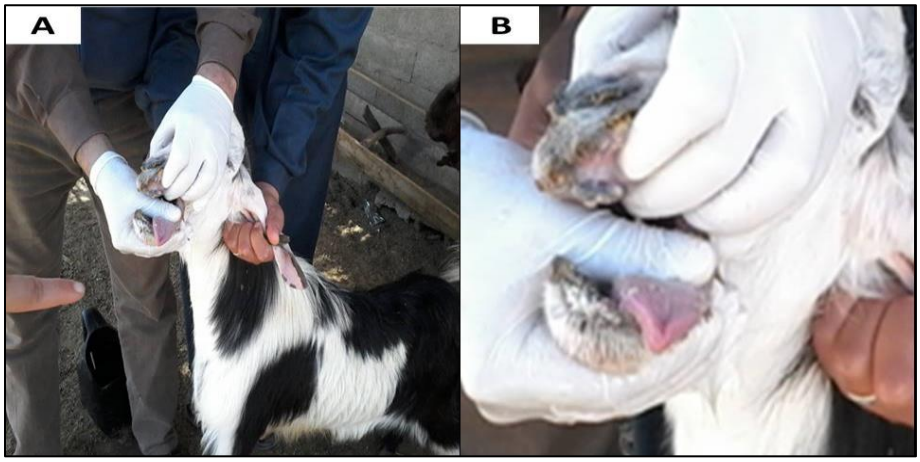

Fig. 2. Development of cyanotic tongue (bluetongue) of a goat clinically affected by BTV. (A) Lesions in a goat. **(B)** Magnified picture of the lesions.

3.2. Serological assay

Using optical wavelength 450 nm, the result was recorded using an ELISA plate reader where positivity/negativity was calculated using the provided equation in the kit. The resultant percentages of positivity were 12.3%, 77.8%, 33.3% and 46.3% for goats/total, goats/total goats, Baladi sheep/total and Baladi sheep/total Baladi sheep respectively.

3.3. Isolation and concentration of BTV

3.3.1. Primary virus isolation using yolk sac route

The isolated buffy coat was inoculated via intra yolk (IY) route in 10-day-old SPF-ECEs and the number of inoculated eggs was according to survival rate with a total of 9 passages. As noticed, the embryos were hemorrhagic cherry red which is a very common feature of the virus **(Fig. 3)**.

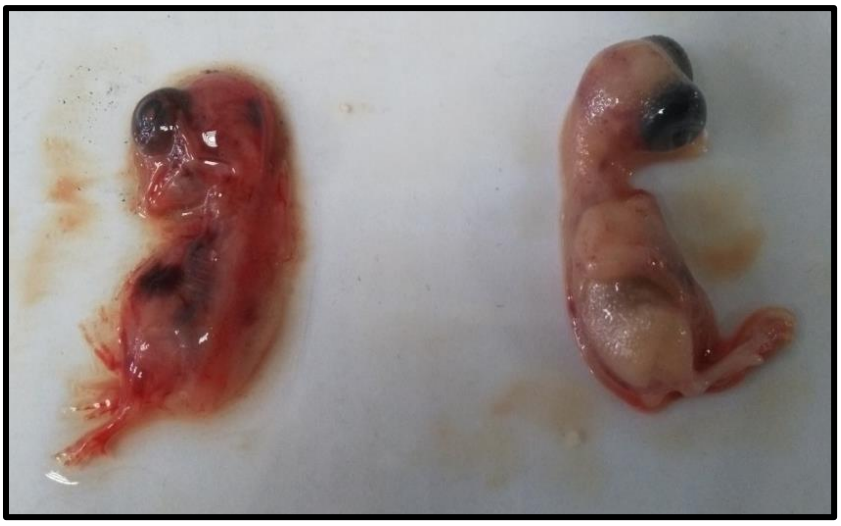

Fig. 3. Hemorrhagic cherry red chicken embryo on the left and control embryo on the right.

3.3.2. Primary virus isolation using CAM route

Nine passages were previously performed using the yolk sac route, and then pock lesions on CAM were observed at 3rd passage after using homogenate of the 9th passage for inoculation via CAM route **(Fig. 4)**. This route was used as a means of confirmation of the presence of the virus.

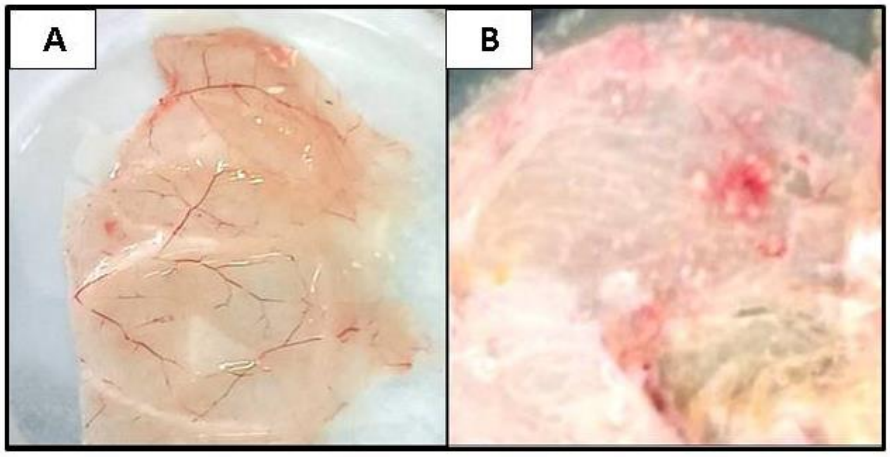

Fig. 4 Pock lesions on CAM of 12th passage SPF-ECE, inoculated at 11 days old, collected at 3rd day PI. Photo A (normal CAM). Photo B (infected CAM).

3.3.3. BTV concentration and partial purification

PEG-6000 was precipitated by high-speed centrifugation. The supernatant was enriched by a partially purified virus and the virus might be concentrated to about 100 folds.

3.3.4. Secondary virus isolation using BHK-21 cell line

The tissue homogenate from embryos infected with the isolated virus undergone several steps of filtration, using qualitative filter papers (11, 9, and 7 cm) and 0.45 µm syringe filters. Six passages were accomplished, but no clear CPE was visualized till the 4th passage. The most common CPE found was rounding of the cells, syncytium formation, and retractile cells with clear necrosis of the nucleus after 48 hours of incubation **(Fig. 5 and 6)**.

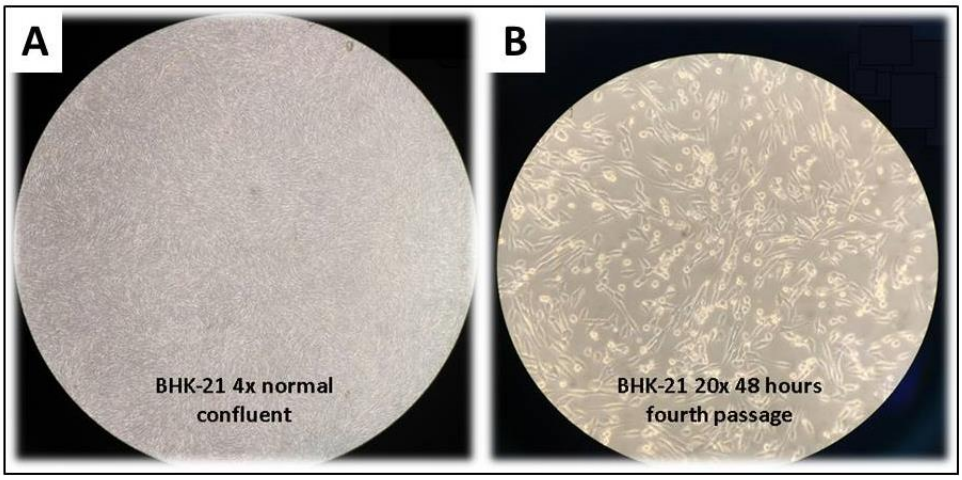

Fig. 5. Tissue culture cell line (BHK-21) (A) Normal confluent cell layer, magnified at 4x, 4th passage. **(B)** CPE is shown as foci of rounding and refractile cells, magnified at 20x, after 48 hours PI, 4th passage.

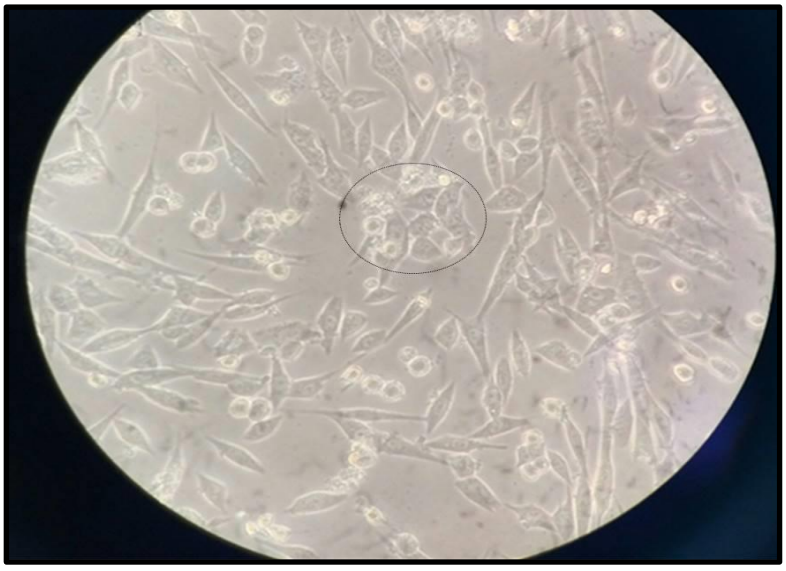

Fig. 6 Tissue culture cell line (BHK-21) showing syncytium formation (marked with circular selection), magnified at 40x, 4th passage, 48 hours PI

3.4. BTV titration (TCID50 and SNT)

$TCID_{50}$ is one of the most widely used methods for virus titration and Reed-Muench is also one of the well-known methods tailored to help in such interpretation and calculation was performed according to Reed and Muench **(Reed & Muench, 1938)** where tissue culture infective dose 50 ($TCID_{50}$) was approximately 10^{-7} $TCID_{50}$/ml. The SNT was used as a qualitative means to further confirm the presence of the infectious BTV particles.

3.5. BTV electron microscopy

3.5.1. Virus concentrate

A negative staining technique was accomplished with phosphotungstic acid to aid in visualizing the virus by TEM. This technique was performed in Analysis and Scientific Services – NRC. The size of the virus ranged from 45 to 56 nm. The BTV distinctive three-concentric layered capsid (outer core, core, and inner core) was detected by TEM **(Fig. 7)**.

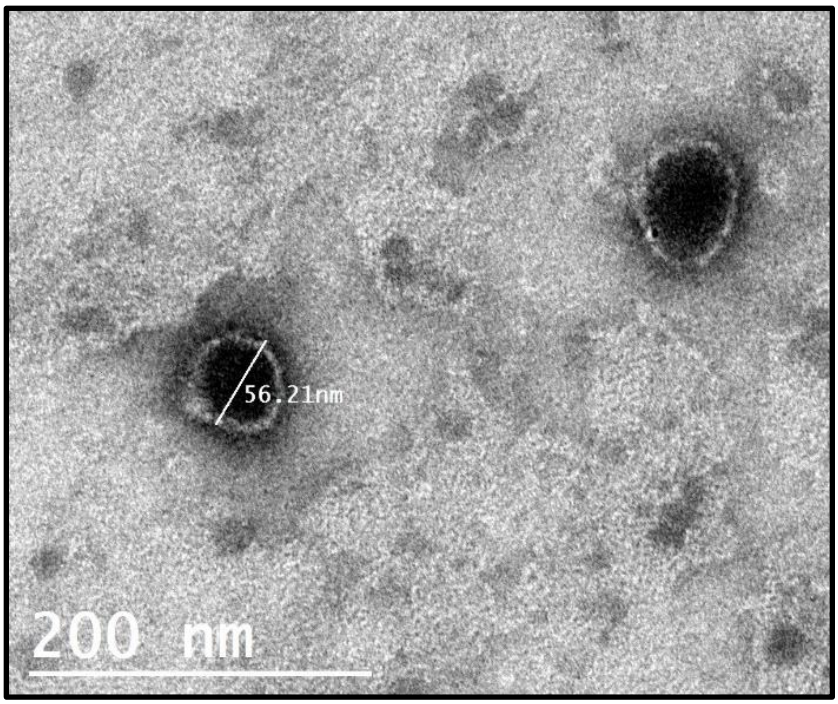

Fig. 7 Negatively-stained electron micrograph demonstrating BTV particles.

3.5.2. Pock lesion on CAM

For further confirmation of identity, the CAM (pock lesion) was taken and processed to be visualized using TEM, where viral particles observed again were in the same previously observed range (40s-50s nm) **(Fig. 8)** similar to TEM performed previously on virus concentrate **(Fig. 7)**.

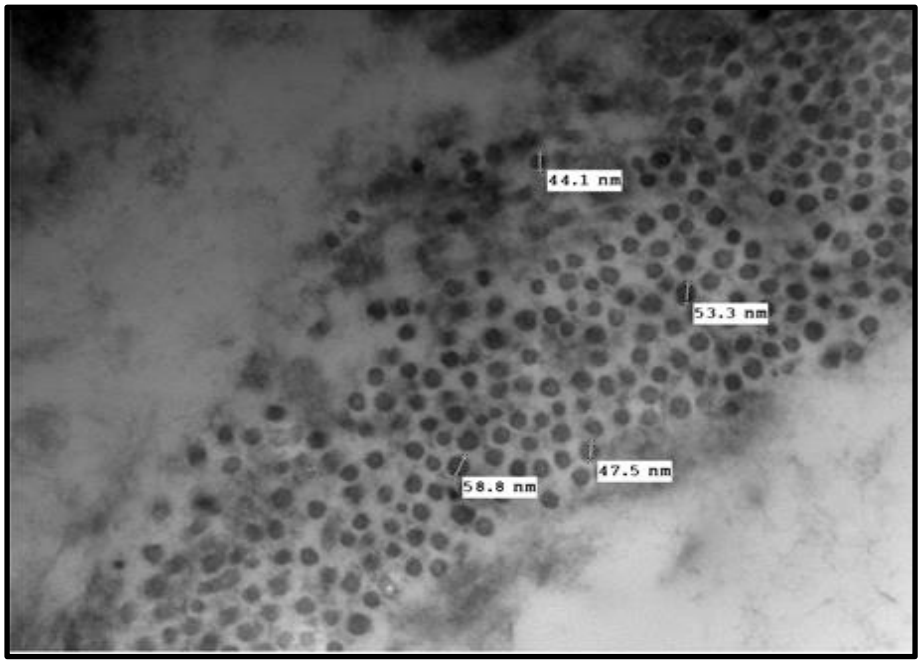

Fig. 8. TEM of CAM tissue depicting a large collection of BTV viral particles.

3.6. BTV immunoelectron microscopy

After incubation of the virus concentrate with control positive antiserum, the reaction was observed under TEM. The aggregation of antibodies and virus particles together was observed (immune aggregation).

3.7. Shotgun proteomic analysis

The proteomic analysis revealed the presence of VP1 protein (signified by several peptides) as shown in **Table 1.**

4. Discussion

BTV is a notifiable emerging disease, which is and will always be a worldwide problem to the industry of domestic animals e.g. sheep (the main host), goats, cattle, and other wild animals. The prevalence of the disease as an arthropod-borne virus; transmitted mainly by various members of genus *Culicoides*; and its predominance in the temperate and tropical areas is directly linked with insect population abundance. Its emergence is due to the nature of the virus (RNA) and the multiplicity of its segments (10) and the disclosure of the 29th serotype **(Wright, 2014)** is a robust evidence in that regard. Outbreaks of BT often lead to devastating economic impact due to direct and indirect losses involving treatment, diagnosis, control measures, and restriction of trade in infected areas. Morbidity, mortality, reduced milk production, early culling, weight loss, reproductive losses, abortions, stillbirths, decreased fertility in males, and lower birth weights are responsible for direct losses **(Rushton and Lyons, 2015)**.

Samples distribution of goats, Baladi sheep, and cattle are shown in Wadi Al-Tur, while samples distribution of goat and cattle are shown in Al-Jubail presenting the distribution mainly of goats because of the abundance of this species and is the main domesticated animal in southern Sinai and the relative scarcity of sheep and cattle there. The team examined many animals but took only samples from suspected cases showing signs of

illness. The limitation of the number of sampled animals is due to the commitment to scheduled visits to certain areas during those field trips, arranged by the national authorities according to the South Sinai regional development program (SSRDP), the degree of incidence encountered, and even important, the reluctance of many owners to allow for blood sampling. In addition, the team had a very strong suspicion that the collected samples are positive because of the prolific numbers of mosquitoes at that time. Samples distribution of Baladi and Merino Sheep in El-Mounib abattoir and Giza Suburbs were illustrated. Regarding sampling in El-Mounib abattoir and Giza Suburbs, the majority of animals sampled in the abattoir were sheep due to the abundance of this animal species, especially that the team did that during Eid El-Adha. Baladi sheep and Merino sheep were examined and only symptomatic animals were sampled. The majority of animals sampled were, of course, Baladi sheep, due to its popularity and existence in large numbers and Merino is vice versa. As noticed, there was no sampling from goats as it's rare to be transferred and slaughtered in the abattoir especially during Eid El-Adha and cattle were totally in a separate ward. The species examined, sampled numbers and distribution of animals were summarized in **Fig. 1** of Bar Chart. An interesting point is that among the 29 **(Wright, 2014)** well-documented BTV serotypes; only the 25th **(Hofmann *et al.*, 2008)** and 27th **(Schulz *et al.*, 2016)** were isolated from goats, which are usually asymptomatic and so the virus isolated in this study is rather unique.

After the initial examination, sheep (Baladi and Merino), goat, and cattle herds pooled serum samples testing was undertaken using BTV VP7 Antibody Test Kit (IDEXX Bluetongue Competition), where the positivity among total tested goats was 77.8% and it was 46.3% among total Baladi sheep tested. The same kit was used for surveillance of sick sheep and goats in different Egyptian governorates as Al-Daqhaliya, Al-Qalyoubiya, and Al-Sharqiya **(Zaher, 2012)** while Biological Diagnostic Supplies Ltd., Surrey, UK (BDSL) was used for serosurveillance of sheep, goats and cattle and results were 23.5%, 10.9%, and 10.7% respectively in Gharbia, Alexandria, Kafr El-Sheikh, Menoufia and Beheira governorates **(El-Bagoury *et al.*, 2013)**. The same previously mentioned kit was used where sheep was 45% and goats were 37% in Giza governorate and sheep 80% and goats 55% in Beni Suef governorate **(Mahmoud *et al.*, 2017)**. On the other hand, when competitive ELISA commercial kit, Veterinary Medical Research & Development (VMRD, Inc. USA) was used to test apparently healthy cattle, sheep, and goats of both sexes all year round in Egypt from El-Beheira, Alexandria, and Matrouh governorates, the overall percentages were as follows:- 58.6% in sheep, 38.1% in goats and 38.9% in cattle **(El-Fayoumy & Bastawecy, 2006)** and when used for screening of goats, sheep and camels from Shalatin, Abo-Ramad and Halayb in Red Sea governorate; the seropositivity was for sheep 33.44%, goats 34.81% and camels 27.97% **(AbouElnaga, 2012)**.

Conventional/traditional diagnosis, through virus isolation, is achieved by primary isolation on ECE via intra-yolk (IY) or intravenous (IV) route, which is deemed to be approximately 1000 times more sensitive. In this study, we used two methods for inoculation in SPF-ECE; the first one is IY, which matches the method described by **Mertens *et al.* (2009)** and **Clavijo *et al.* (2000)** and that led to cherry red embryo **(Gers *et al.*, 2016)** observed in **Fig. 3** at 9th passage in SPF-ECE at 4 days PI; the second route being CAM, which is scarce in usage for BTV isolation, found rarely in publications **(Gray & Bannister, 1961; Cherrington *et al.*, 1985a)** without clear resultant pock lesion formation, which was observed in this study **(Fig. 4)** at 3rd passage 3 days PI (inoculated from homogenate obtained from 9th passage with the appearance of a cherry red embryo). Pock lesion formation was observed in Family *Reoviridae*, Genus *Orthoreovirus* **(Kalupahana, 2017)**, but not in Genus *Orbivirus*.

After isolating the BTV virus primarily on SPF-ECE, there was a necessity to concentrate and partially purify the virus to be able to identify the virus morphologically by electron microscopy. The method used in this study is PEG-6000 with high-speed centrifugation which was originally invented by **Yamamoto *et al.* (1970)** and is a much gentler method than other described concentration and purification methods as ultracentrifugation and molecular sieve filtration, which can cause severe virus loss when resuspended **(Killington *et al.*, 1996; Alves, 2015)**.

After undergoing the concentration step of BTV using SPF-ECE tissue homogenate, the revelation of the virus identity was achieved using electron microscopy, specifically TEM, as shown in **Fig. 7** with an average diameter of 50 nm noticeably matching morphological observations by **(Mertens *et al.*, 2004; Zaher, 2012)** using same negative staining technique with phosphotungstic acid, but with the difference in average diameter of 86 nm. The same technique was used for CAM with pock lesions, via TEM, after tissue processing, to further confirm the presence of the virus, where it was again identified with an average diameter of 50 nm **(Fig. 8)**. Furthermore, TEM was used in this study due to its wide usage, popularity, and functionality in the field of virology. In conjunction with TEM, negative staining has been established for over 60 years as a fast, robust, and universal EM technique and still plays an essential role in this field **(Richert-Poggeler *et al.*, 2018)**.

Two main types of IEM are known; immunoaggregation and immunolocalization. Immunoaggregation essentially involving the use of antibodies and can be termed also unlabeled immunoelectron microscopy, in which the virus is mixed with virus-specific antiserum and centrifuged at medium speed, and when the antibody recognizes the virus, an aggregation of viral particles is formed in the immune complex, thus concentrating and specifically identifying the particles. The immunoaggregation was observed in the current study.

Secondary isolation of BTV on cells, either mammalian such as BHK-21 or Vero cells; or insect cell cultures such as KC cell and C6-36 is a well-documented and accepted practice **(Mertens *et al.*, 2009; Krishnajyothi *et al.*, 2016)**. The same approach was adopted in this study, where secondary isolation was performed on BHK-21 **(Fig. 5 and 6)** with clear CPE observed at 4th passage 2 days PI matching results by**Chauhan *et al.* (2014)**. Another approach is to isolate BTV primarily on ECE or KC cells followed by BSR cells **(Verdezoto *et al.*, 2018)**, directly on BHK-21 **(Chauhan *et al.*, 2014)**, ECE **(Balaro *et al.*, 2014)**, KC cells **(Veronesi *et al.*, 2013)** or KC cells followed by Vero cells **(Lorusso *et al.*, 2018)**.

As a final effort using proteomic approach for confirmation for the presence of BTV virus, shotgun proteomic analysis was conducted using the obtained virus concentrate elucidating the presence of VP1 protein **(Table 1)** and thus verifying the existence of the virus among the obtained samples. This proteomic approach is rarely used in identification of viruses generally. The investigations conducted worldwide regarding BTV using shotgun proteomic analysis are limited to quantitative and not qualitative studies via BTV-infected cells **(Du *et al.*, 2016; Mohl *et al.*, 2017)** and as to authros' knowledge, this is the first time for this method to be used for BTV diagnosis in Egypt and worldwide.

5. Conclusion

In this study, we proved the existence of BTV in the clinical field specimens through multiple steps of virus isolation, concentration, titration, serology, electron microscopy, and immunoelectron microscopy. This was followed by confirmatory shotgun proteomic analysis revealing the occurrence of VP1 protein and thus verifying undeniably the presence of Bluetongue virus in the Egyptian field during year 2016 in Egypt.

6. Acknowledgment

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7. Authors Contribution

All authors contributed equally in the planning of the study, drafting the manuscript and all of them approved the final version of this article.

8. Conflict of interest

The authors declare no conflict of interest.

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