Ivermectin also inhibits the replication of bovine respiratory viruses (BRSV, BPIV-3, BoHV-1, BCoV and BVDV) in vitro

Kadir Yesilbag *, Eda Baldan Toker, Ozer Ates

Department of Virology, Bursa Uludag University, Faculty of Veterinary Medicine, 16059, Bursa, Turkey

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ABSTRACT

Bovine respiratory disease (BRD) complex is an important viral infection that causes huge economic losses in cattle herds worldwide. However, there is no directly effective antiviral drug application against respiratory viral pathogens; generally, the metaphylactic antibacterial drug applications are used for BRD. Ivermectin (IVM) is currently used as a broad-spectrum anti-parasitic agent both for veterinary and human medicine on some occasions. Moreover, since it is identified as an inhibitor for importin α/β-mediated nuclear localization signal (NLS), IVM is also reported to have antiviral potential against several RNA and DNA viruses. Since therapeutic use of IVM in COVID-19 cases has recently been postulated, the potential antiviral activity of IVM against bovine respiratory viruses including BRSV, BPIV-3, BoHV-1, BCoV and BVDV are evaluated in this study. For these purposes, virus titration assay was used to evaluate titers in viral harvest from infected cells treated with non-cytotoxic IVM concentrations (1, 2.5 and 5 μM) and compared to titers from non-treated infected cells. This study indicated that IVM inhibits the replication of BCoV, BVDV, BRSV, BPIV-3 and BoHV-1 in a dose-dependent manner in vitro as well as number of extracellular infectious virions. In addition, it was demonstrated that IVM has no clear effect on the attachment and penetration steps of the replication of the studied viruses. Finally, this study shows for the first time that IVM can inhibit infection of BRD-related viral agents namely BCoV, BPIV-3, BVDV, BRSV and BoHV-1 at the concentrations of 2.5 and 5 μM. Consequently, IVM, which is licensed for antiparasitic indications, also deserves to be evaluated as a broad-spectrum antiviral in BRD cases caused by viral pathogens.

1. Introduction

Bovine respiratory disease (BRD) complex is described as the cause of major significant economic losses in cattle production worldwide. Viral pathogens usually act as the primary cause of BRD (Autio et al., 2007). The most important viral pathogens responsible for BRD include Bovine respiratory syncytial virus (BRSV; *Bovine orthopneumovirus*), Bovine parainfluenza virus type 3 (BPIV-3; *Bovine respirovirus-3*), Bovine herpesvirus type 1 (BoHV-1), Bovine coronavirus (BCoV) and Bovine viral diarrhea virus (BVDV, *Bovine pestivirus A* and *B*) (Autio et al., 2007; Urban-Chmiel and Grooms, 2012). Since these viral pathogens cause immunosuppression through various mechanisms (Jones and Chowdhury, 2010), the animals become more susceptible to secondary infections (Autio et al., 2007). Some of the mentioned viral agents are not only associated with BRD; for example BCoV also causes winter dysentery which is associated with diarrhea in cattle. Similarly, BVDV infections lead to diarrhea, growth retardation and congenital anomalies.

BoHV-1 is additionally responsible for infection in the nervous, reproductive and digestive systems in cattle. Although there are several different types of vaccines available to prevent these infections (Chamorro and Palomares, 2020), there is no available drug that directly targets therapy for infections caused by these viruses.

BCoV belongs to the *Coronaviridae* family and the *Betacoronavirus* genus. BVDV is in the *Pestivirus* genus of *Flaviviridae* family. Although BRSV and BPIV-3 were previously classified as two sub-families of *Paramyxoviridae*, they were reclassified in two different families, as BRSV is located in the *Pneumoviridae,* while BPIV-3 is in the *Paramyxoviridae* family. BoHV-1 is a DNA virus from *Varicellovirus* genus in the *Herpesviridae*. All of these viruses have the enveloped virions and the viral genome of mentioned viruses is RNA, excluding BoHV-1 (ICTV, 2019). The replications of BCoV, BVDV, BRSV and BPIV-3 occur in the host cell cytoplasm, while BoHV-1 replication occurs in the cell nucleus. The extracellular scattering for BCoV, BRSV, BPIV-3 and BoHV-1 occurs by budding, while maturation of BVDV occurs on intracytoplasmic

* Corresponding author.
E-mail addresses: kyesilbag@uludag.edu.tr (K. Yesilbag), edabaldan@uludag.edu.tr (E.B. Toker).

membranes without evidence of budding.

Eukaryotic cells have a double-membrane nuclear envelope that separates the cytoplasm and the nucleus. This envelope contains large multiprotein channels called nuclear pore complexes (NPCs). Translocation of small proteins can occur via passive diffusion from the NPCs, whereas the translocation of large protein complexes is a process dependent on energy and signaling mechanism through NPCs. Karyopherins are adaptor proteins and they are involved in the transport of large proteins between nucleus and cytoplasm. Karyopherins can act as importins (proteins that get into the nucleus) or exportins (proteins that get out of the nucleus). Nuclear import and export are both tightly-regulated and highly specific process. Nuclear import is regulated by nuclear localization signal (NLS) recognizing importins that interact with nucleoporins of the NPC to transport cargo into the nucleus. Nuclear export is controlled by exportins and the nuclear export signal (NES) of the cargo (Görlich et al., 1995; Tran et al., 2007). There are many studies indicating that viral proteins of some viruses (such as Human immunodeficiency virus-1, Epstein Barr virus, Pseudorabies virus, Herpes simplex virus-1) are transported by importin α/β through the recognition of NLSs in order to escape the immune response by modulating the nuclear traffic, integrating the host genome, and accessing host gene transcription (Kawashima et al., 2013; Lv et al., 2018; Mastrangelo et al., 2012; Raza et al., 2020).

Ivermectin (IVM) is a chemically modified derivative of naturally produced avermectin B1, contained 22.23-dihydro-avermectin B1a (~80 %) and 22.23-dihydro-avermectin B1b (~20 %) (Campbell et al., 1983). IVM is currently used as a broad-spectrum antiparasitic agent against nematodes after both oral and parenteral administration. Significantly, it has also been reported to have in vitro antiviral activity against various viruses in recent years. Initially, it was reported that IVM acts as an inhibitor between the HIV-1 integrase protein and the importin α/β responsible for the transport of this protein to the nucleus (Wagstaff et al., 2012). Then, IVM has been confirmed to inhibit the transport of viral proteins to the host nucleus through inhibition of importin α/β -mediated NLS, which is responsible for the signaling and the transport processes of the host cell (Yang et al., 2020). IVM was reported to reduce viral replication in RNA viruses such as *Retroviridae* (*Lentivirus* genus; HIV-1), *Flaviviridae* (*Flavivirus* genus; Dengue virus, Japanese encephalitis virus, West Nile virus WNV, tick-borne encephalitis virus and Zika virus), *Togaviridae* (*Alphavirus* genus; Chikungunya virus, Venezuelan equine encephalitis virus), *Arteriviridae* (*Betaarterivirus* genus; Porcine reproductive and respiratory syndrome virus) and *Paramyxoviridae* (*Orthoavulavirus* genus; Newcastle virus) (Azeem et al., 2015; Lee and Lee, 2016; Lundberg et al., 2013; Mastrangelo et al., 2012; Varghese et al., 2016; Yang et al., 2020). It has been similarly shown to act as antiviral against the DNA viruses such as *Herpesviridae* (*Varicellovirus* genus; Pseudorabies virus and BoHV-1) and *Circoviridae* (*Circovirus* genus; Porcine circovirus type 2) (Lv et al., 2018; Raza et al., 2020; Wang et al., 2019). Considering the effectiveness of the IVM active ingredient, which came to the fore during the SARS-CoV-2 epidemic, it has become more important to investigate the effectiveness of IVM for viruses that widely affect respiratory tissues. In this study, the potential of antiviral activity of IVM was evaluated against BRSV, BPIV-3, BoHV-1, BCoV and BVDV at different concentrations in vitro. The antiviral potential of IVM was appraised separately for each virus in terms of its effects on the viral attachment, penetration, intracellular replication and shedding of mature virus particles from the infected cells.

2. Material and method

2.1. Cell culture and viruses

Madin Darby Bovine Kidney (MDBK) cell line was used for the propagation and titration of the viruses. MDBK cell cultures were grown at 37 °C, in 5% CO_2 atmosphere using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal calf serum (FCS), 100 UI/mL Penicillin/Streptomycin and 250 µL/mL Amphotericin B solution.

BRSV Atue strain, BPIV-3 SF-4 strain, BoHV-1 Cooper strain, BCoV Mebus strain, and BVDV NADL strain were used for testing antiviral activities of IVM. Infective titers ($TCID_{50}$) for the test viruses were $10^{-6.00}$, $10^{-7.00}$, $10^{-6.75}$., $10^{-6.25}$, and $10^{-5.25}$ respectively. Viruses were obtained from the virus collection at our laboratory.

2.2. Compounds of IVM

A commercial injection solution (Avromec, Topkim, Turkey) containing 10 mg/mL ivermectin active ingredient was used. The active ingredient of IVM was composed of 22.23-dihydro-avermectin B_{1a} (~80 %) and 22.23-dihydro-avermectin B_{1b} (~20 %) components. IVM stock solution was prepared as including 1000 µM IVM and was equilibrated to room temperature. Toxicity and efficiency results were also validated with a pure compound of IVM supplied by another commercial company (Pharmactive, Turkey).

2.3. Cell viability assay

For cell viability assay, 24-well plates were coated with MDBK cells at a concentration of 2×10^5 cells/mL. After 24 h, the cells were treated with 0, 1, 2.5, 5, 10, 12.5, 25, 50 and 100 µM IVM diluted in DMEM for 6 days at 37 °C with 5% CO_2 atmosphere. During that stage, cells from individual wells were collected with 24 h intervals by trypsinization and centrifuged at 1500 rpm for 10 min. Cell pellets were pipetted with 1 mL of DMEM and viability rate (viable cell/total cell counts) was determined by trypan blue staining. Viability of cells were calculated as percentages based on the rate of live cells to total cells.

2.4. Experiment 1: Effect of IVM on virus replication in infected cells

The antiviral activity of IVM was tested using the cells infected with the virus. In this test, a separate 24-well plate was used for each virus. For this purpose, 24-well plates were coated with MDBK cells at a concentration of 2×10^5 cells/mL. After 24 h, cells supernatant was removed and cells were infected by 200 µL of virus suspensions (BRSV, BPIV-3, BoHV-1, BCoV and BVDV) diluted at a concentration of $100TCID_{50}$. At this stage, two rows of cells were not infected with the virus to follow uninfected MDBK cells. After inoculation of viruses, BoHV-1 was incubated for 2 h and other viruses were incubated for 1 h for virus adsorption onto cells. The inoculum was removed and monolayers were washed three times with PBS. Test design on the vertically positioned plates were arranged as follows: Four wells of each column on the plate were used for a defined IVM concentration and, four wells of the rows arranged for a single test period (48 h, 72 h, 96 h, 120 h) (For details visit supplementary material 1). After virus inoculation, individual wells of the columns were treated with 1 mL of DMEM containing 0, 1, 2.5 and 5 µM IVM, respectively. In that design, the first well of the row (0 µM IVM) served as non-treated control while 3 wells were treated with non-cytotoxic IVM concentrations (1, 2.5 and 5 µM) for every sampling period. Additionally, in order to follow non-infected cells during the experiment, one row of the non-infected MDBK cells was treated with IVM free-DMEM and the other row with DMEM containing 5 µM IVM (see supplementary material 1 and 2 for test design and applied steps). DMEM used for this step of the experiment was not supplemented with FCS. The test plates were incubated for 5 days. At defined sampling periods (48 h, 72 h, 96 h, 120 h pi), culture supernatants and virus-infected cells in the wells treated with 0, 1, 2.5 and 5 mM IVM were collected together by freeze-thawing, and stored at -80 °C until virus titration assay.

2.5. Experiment 2: Effect of IVM on virus attachment to host cell

To evaluate the effect of IVM on virus binding to the target cells, each of the viruses (100TCID$_{50}$) treated with 5 μM IVM was incubated at 37 °C for 1 h in the test tube. The culture media on MDBK cells prepared in 24-well plates on the day before testing were removed. Then, 200 μL of the virus suspension treated with IVM was added to the layered MBDK cells. Just after the virus inoculation, test plates were incubated at 4 °C for 1 h. The same procedure was simultaneously applied to non-treated viruses as the indicator for non-blocked virus attachment. After incubation, infected cell surfaces were washed three times with PBS. One milliliter of IVM free-DMEM was added onto the cells and incubated at 37 °C and 5% CO$_2$ atmosphere until the 80 % CPE developed in the non-treated virus control cells. The culture supernatants and virus-infected cells were collected together by freeze-thawing and subjected to virus titration assay.

2.6. Experiment 3: Effect of IVM on virus penetration to host cell

The cell culture media of MDBK cells prepared in 24-well plates on the day before testing were removed. Two hundred microliters of virus suspensions at 100TCID$_{50}$ concentration were added onto the MDBK cell monolayers. Virus inoculated cultures were incubated at 4 °C for 1 h. The infected cell surfaces were washed three times with PBS. Subsequently, DMEM containing 5 μM IVM was added onto the cells and incubated at 37 °C for 30 min. Simultaneously, IVM free-DMEM was added onto infected cells prepared as virus control. The infected cell surfaces were washed three times with PBS (pH 3.00). One milliliter of FCS free DMEM was added onto the cells and incubated at 37 °C, 5% CO$_2$ atmosphere until the 80 % CPE developed in the virus control cells. The culture supernatants and virus infected cells were collected together by freeze-thawing and subjected to virus titration assay.

2.7. Experiment 4: Effect of IVM on the number of extracellular infectious virions

The culture media from the infected cells (not including the cell debris) were used to evaluate the effect of IVM on the number of extracellular infectious virus particles shed into culture media. For this purpose, virus inoculation and IVM treatments were applied, as described in the procedure for Experiment 1 (the antiviral activity of IVM). The cell cultures infected with the viruses were incubated for 5 days at 37 °C, 5% CO$_2$ atmosphere. The plates were examined daily for CPE development. The culture supernatants, but not the infected cells, were collected at every 24 h after 2nd days of infection and stored at -80 °C until virus titration assay.

2.8. Virus titration assay

Whether IVM inhibits virus growth indicating antiviral effect was tested by infective virus titration assay. For this purpose, the TCID$_{50}$ titers obtained in IVM-treated infected cells and IVM non-treated infected cells were compared. The virus titration assay was simultaneously applied to virus infected cells treated with IVM and non-treated infected cells for each virus. Infectious titers were determined by 10-fold (Log 10) serially dilution method in DMEM. In the test protocol, four parallel columns in 96-well microplates were used for each step of virus dilution. Infected and non-infected wells were also used as positive and negative test controls, respectively. MDBK cell suspension (2 × 10^5 cells/mL) was added in all the test wells and incubated at 37 °C, 5% CO$_2$ atmosphere during the experiment. CPE developments were recorded daily by an inverted light microscopy. TCID$_{50}$ titers were calculated according to the Spearman-Kàrber method.

2.9. Statistical analysis

Mann-Whitney *U* test was applied for statistical evaluation of the antiviral efficiency led by IVM. For this purpose, IBM SPSS Statistics 20 (Chicago, IL, USA) package program was used. The statistical significance values were defined as P < 0.05.

3. Results

3.1. Non-cytotoxic concentrations of IVM on MDBK cells

Cytotoxicity of IVM was evaluated as following the effect of various IVM concentrations on the viability rate of the cultured MDBK cells. As a result of viable cell count to determine the cytotoxicity of different IVM concentrations (1, 2.5, 5, 10, 12.5, 25, 50 and 100 μM) on MDBK cells, IVM concentrations of 10 μM and above were determined to be toxic. On the 144th hour of the experiment, cell viability rates for non-treated cells (0 μM) and 1, 2.5, 5 and 10 μM IVM treatments were 96.08 %, 94.94 %, 94.12 %, 93.51 % and 0.0 % respectively (Fig1). Therefore, 1, 2.5 and 5 μM concentrations of IVM were selected to evaluate the antiviral activity of IVM.

3.2. Inhibition of BRSV, BPIV-3, BoHV-1, BCoV, and BVDV replications by IVM

To test whether IVM has antiviral activity against BRSV, BPIV-3, BoHV-1, BCoV, and BVDV propagation, the viruses were inoculated on the MDBK cells. The cells were treated with IVM at different concentrations. The CPE formations in infected cells were recorded daily using an inverted light microscope (Fig. 2). Both infected cells and the culture media were collected and progeny virus titer was determined by the virus titration assay at 48, 72, 96 and 120th pi. There was no significant decrease in detected virus titers in cultures treated with 1 μM IVM compared to titers from non-treated infected cells. In the presence of 2.5 and 5 μM IVM, the virus titers were decreased by one and three logs for BRSV (Fig. 3A), one and two logs for BPIV-3 (Fig. 3B), three and four logs for BoHV-1 (Fig. 3C), one and three logs for BCoV (Fig. 3D) and three and four logs for BVDV (Fig. 3E), respectively. On the 5th day post infection, compared to non-treated infected cultures, there was 99.94 %, 94.38 %, 99.99 %, 100 % and 99.99 % inhibition in titers for BRSV, BPIV-3, BoHV-1, BCoV and BVDV replication for the groups treated with 5 μM IVM, respectively. For BPIV-3, BVDV and BoHV-1, a statistically significant decrease was found between viruses treated with 2.5 and 5 μM IVM and non-treated controls. On the other hand, a significant statistical difference was determined for viruses treated only with 5 μM IVM for BCoV and BRSV (P < 0.05).

3.3. Effect of IVM on virus attachment and penetration

Whether IVM affects the attachment of viruses to the cell membrane and penetration into the host cell was also evaluated by experiments #2 and #3. The attachment and penetration test results indicated a statistically insignificant difference in virus titers between IVM-treated and non-treated viruses (Table 1).

3.4. Inhibition of viral shedding by IVM

To determine whether IVM inhibits the number of extracellular infectious virions, virus titration assay was applied to culture supernatants obtained from wells infected with BRSV, BPIV-3, BoHV-1, BCoV and BVDV. It was determined that 1 μM IVM did not suppress the shedding amount of mature virus particles for the mentioned viruses. For BRSV and BPIV-3, in the presence of 5 μM IVM, the virus titers were decreased by nearly four and two logs, respectively (Fig. 4A, **B**). In the presence of 2.5 and 5 μM IVM, the virus titers were decreased by nearly two and three logs for BCoV and three logs for BoHV-1, respectively (Fig. 4C, **D**).
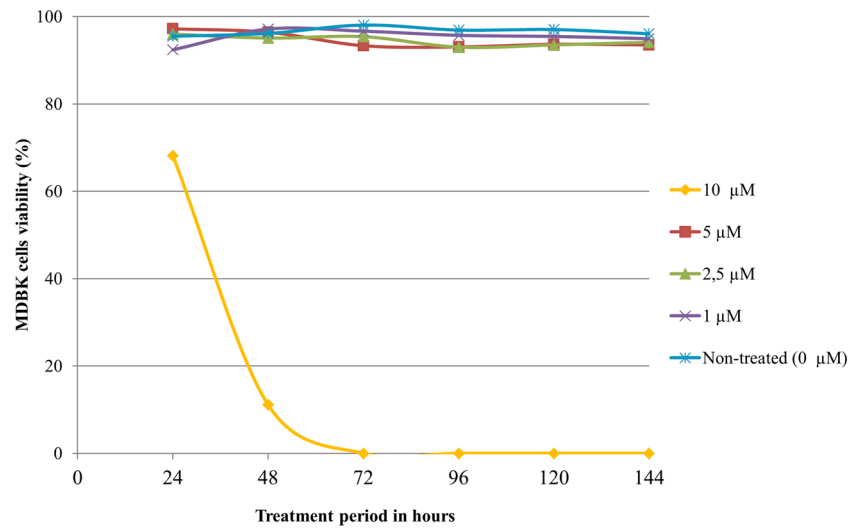
Fig. 1. The percentage of cell viability of the MDBK cells treated with various concentrations of ivermectin during 6 days. Cell viability rates were estimated by live cell counting after staining.
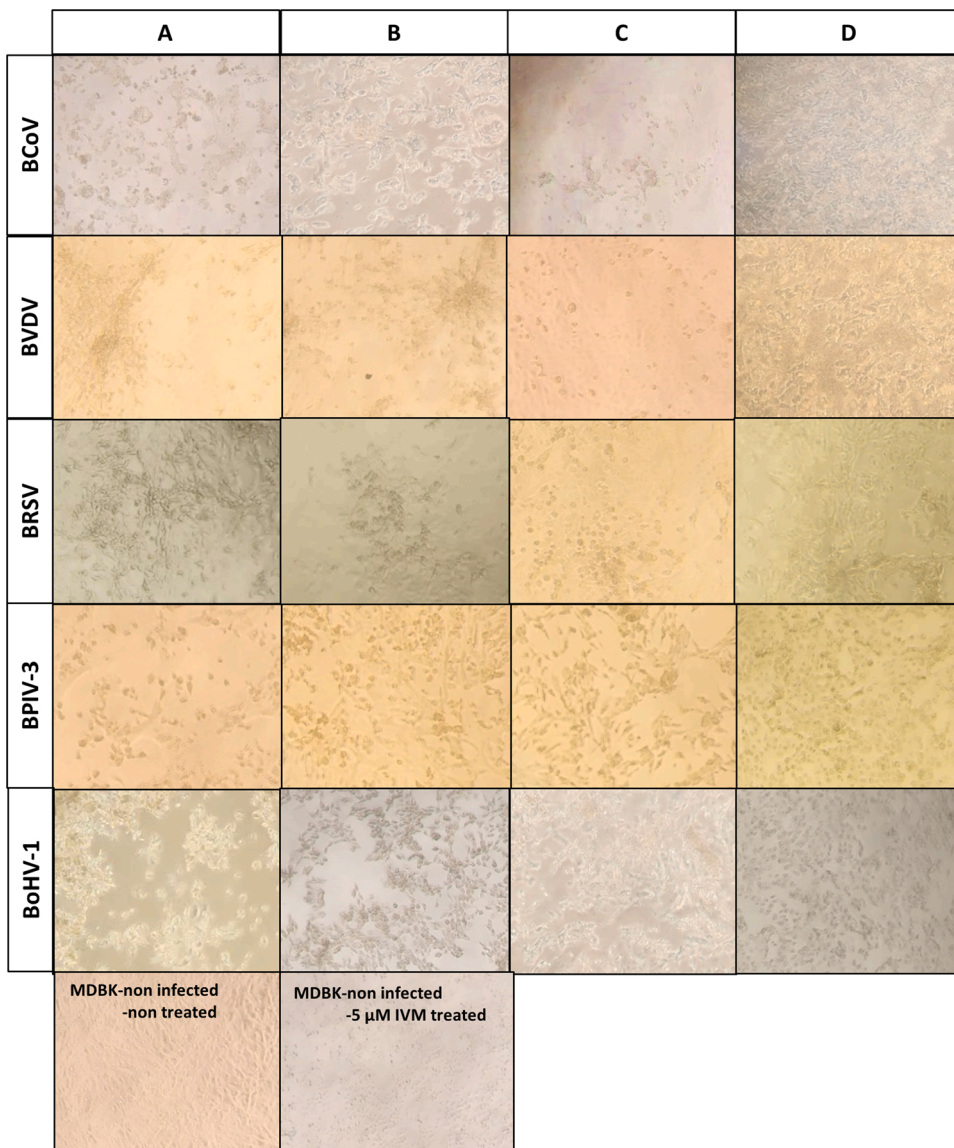

Fig. 2. Microscope images obtained in virus infected non-IVM treated MDBK cells and infected cells treated with IVM on 4th day pi (x20 magnification).

Effect of various IVM concentrations on replication of the test viruses on MDBK cell culture: A. Virus infected cells with 0 μM IVM; B. Virus infected cells with 1 μM IVM; C. Virus infected cells with 2.5 μM IVM; D. Virus infected cells with 5 μM IVM.

Infected cultures were incubated in 37 °C incubator with/without IVM treated DMEM, screened daily for virus propagation and imaged on day 4th pi.
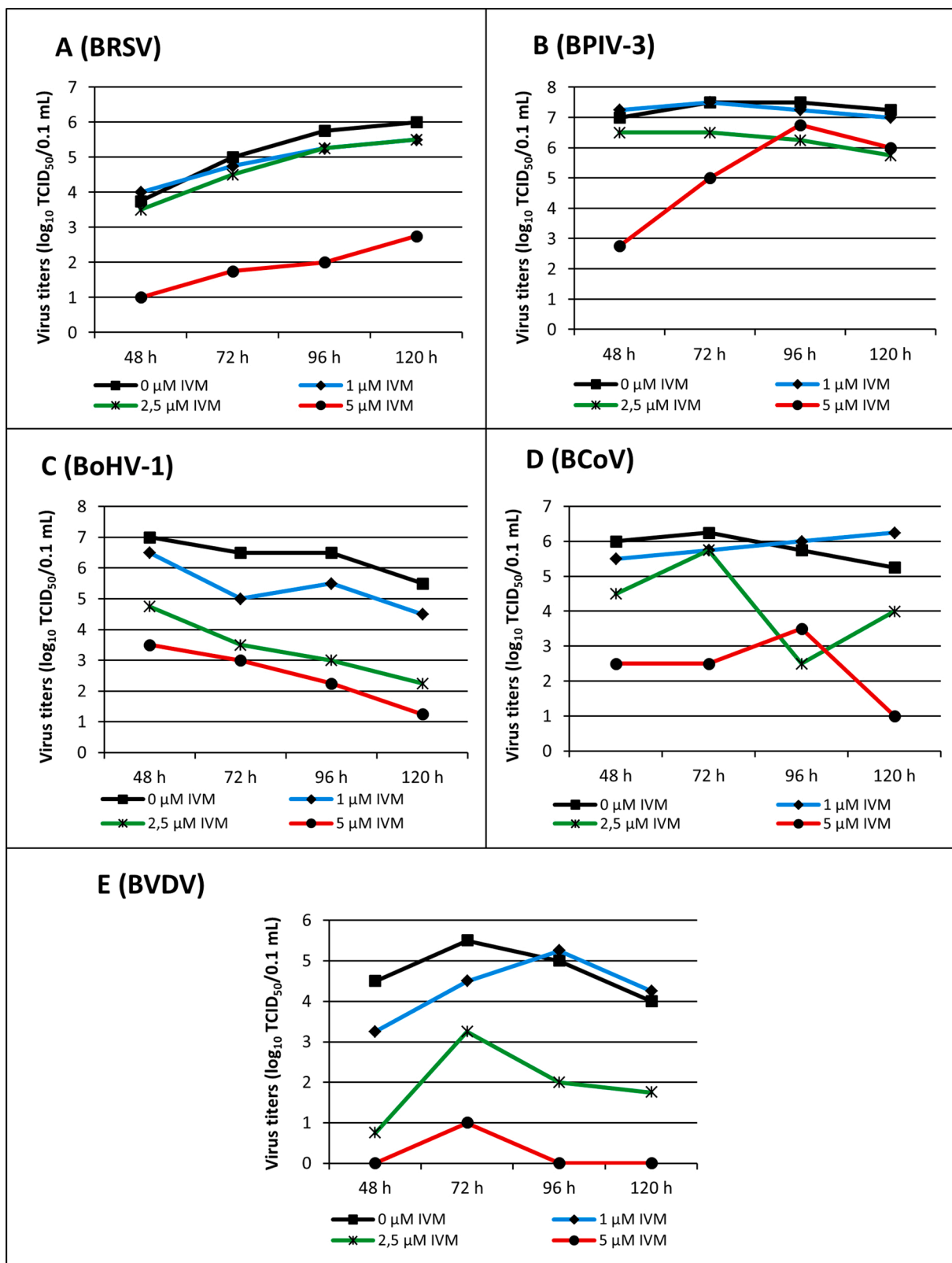
Fig. 3. Effect of various doses of ivermectin treatment on replication kinetics of bovine respiratory viruses in vitro.
Infected MDBK cell culture was treated with DMEM containing 0, 1, 2.5 and 5 μM ivermectin. Samples were collected from individual wells as including infected cells and culture media at defined periods with 24 h intervals. Detected virus titers are presented as $\log_{10}$ TCID$_{50}$.

Table 1
Infective titers of viruses either non-treated or treated with IVM for detecting the effects on the attachment (Experiments #2) and penetration (Experiments #3).

Experiment no	Target	Treatment	Viral titers (Log10 TCID$_{50}$)*				
			BRSV	BPIV-3	BoHV-1	BCoV	BVDV
#2	Virus attachment	*Non-treated*	$10^{6.75}$	$10^{7.00}$	$10^{5.75}$	$10^{4.50}$	$10^{5.50}$
		IVM-treated	$10^{6.75}$	$10^{6.75}$	$10^{5.50}$	$10^{4.50}$	$10^{5.50}$
#3	Virus penetration	*Non-treated*	$10^{5.50}$	$10^{6.50}$	$10^{5.75}$	$10^{4.75}$	$10^{3.50}$
		IVM-treated	$10^{5.25}$	$10^{6.50}$	$10^{5.25}$	$10^{4.25}$	$10^{2.75}$

* No statistical significance was observed between titers obtained from treated and non-treated experiments of the particular virus species.

A three logs reduction was recorded in titer for BVDV from the culture treated with 2.5 μM IVM, while the virus growth was totally blocked by adding 5 μM IVM to the culture media (Fig. 4E). On the 5th day post infection, compared to non-treated infected cultures, there was 99.99 %, 94.38 %, 99.99 %, 96.84 % and 100 % inhibition in titers for viral shedding of BRSV, BPIV-3, BoHV-1, BCoV and BVDV for the groups treated with 5μM IVM, respectively. For BoHV-1 and BVDV, a statistically significant decrease was found between viruses treated with 2.5 and 5 μM IVM by comparison to non-treated virus controls. On the other hand, a significant statistical difference was determined for BPIV-3, BCoV and BRSV treated only with 5 μM IVM (P < 0.05).

4. Discussion

The vaccination and metaphylactic antibacterial applications are the most widely used methods to reduce the incidence and losses due to BRD. Considering that viral pathogens (i.e. BRSV, BPIV-3, BoHV-1, BCoV and BVDV) are the primary agents in BRD cases, treatment studies that can reduce the replication and shedding of responsible viruses through antiviral efficiency can be evaluated as an adjunct approach. This approach may result not only in a decrease in the use of antimicrobials but also in an increase in animal welfare. After the global pandemic of COVID-19, the possible antiviral capacity of current therapeutics is widely re-visited. Considering the fact that IVM can inhibit the replication of SARS-CoV-2 (Caly et al., 2020), the studies evaluating the antiviral effect of IVM have come to the fore again.

In this study, possible antiviral efficiency of IVM against important viral agents (BRSV, BPIV-3, BoHV-1, BCoV and BVDV) threatening bovine respiratory health was evaluated at non-toxic concentrations of 1, 2.5 and 5 μM detected by viability assay. It was determined that IVM does not prevent attachment and penetration of the mentioned viruses into the host cell. Similarly, IVM has been previously reported to be ineffective on cell binding and penetration of BoHV-1 (Raza et al., 2020). On the other hand, when evaluating intracellular replication and mature virus particle scattering for BRSV, BPIV-3, BoHV-1, BCoV and BVDV, a various level of decrease in virus titers was detected. These data show that IVM has an inhibitory mechanism of effect after the virus entry into the cells.

In the experiment, an approximately 10^2 to 10^5 reduction in virus titers was detected for BCoV both in the replication (experiment #1) and the number of extracellular infectious virions (experiment #4) in terms of in the presence of 5 μM IVM. There are no previous studies showing antiviral efficiency of IVM for BCoV replication. Significantly, SARS-CoV-2 and BCoV are located in the *Betacoronavirus* genus. Caly et al. (2020) reported an approximately 5000-fold reduction in viral RNA replication at 48 h after the treatment with 5 μM IVM in Vero/hSLAM cells infected with SARS-CoV-2. Similar results were obtained in our study in that BCoV at all the sampling periods of 48, 72, 96 and 120 h pi. During the viral replication stage of coronaviruses, the cargo protein in the cytoplasm binds to importin α/β and transfers it to the nucleus via the NPC (Caly et al., 2020). These data can also suggest that IVM prevents importin α/β from binding to viral protein and entering the nucleus in betacoronaviruses.

For BVDV replication (experiment #1), an approximately three and four logs decrease of viral titer was found in the presence of 2.5 μM and 5

μM IVM, respectively. Significantly, no viral titer was detected in BVDV infected cell supernatant (experiment #4) with 5 μM IVM treatment. Therefore, IVM was evaluated to stop the shedding of BVDV. Several studies are reporting that IVM can inhibit replication of few human pathogen flaviviruses including Zika virus, Dengue virus, West Nile fever and Yellow fever virus (genus *Flavivirus* in *Flaviviridae*) (Ji and Luo, 2020; Mastrangelo et al., 2012; Tay et al., 2013; Wagstaff et al., 2012; Yang et al., 2020). Therefore, the current study represents the first data on the total inhibition of a virus species in the *Pestivirus* genus of *Flaviviridae*. It was shown in the previous studies that IVM blocks the nuclear transport of nonstructural protein 5 (NS5), which is essential for viral RNA replication of HIV-1 and Dengue virus (Tay et al., 2013; Wagstaff et al., 2012). Also, IVM caused a 60 % reduction in NS5 levels in the nucleus during Zika virus replication (Ji and Luo, 2020). IVM can also act as a highly specific inhibitor of intracellular viral RNA synthesis by targeting NS3 helicase activity (Mastrangelo et al., 2012). NS5 and NS3 are two important non-structural proteins and they are also found in pestiviruses infected cultures (Tautz et al., 2015). Therefore, although it is matter of further research, IVM could possibly inhibit BVDV replication through similar mechanisms.

For BRSV, the viral titers were detected to reduce three and four logs by 5 μM IVM treatment in experiment#1 and experiment#4 compared to non-treated infected cells. For BPIV-3, an approximately two logs reduction in virus titer was found in the presence of 5 μM IVM in experiment#1 and experiment#4. Both BRSV and BPIV-3 share a similar mechanism of pathogenesis including asymptomatic infection localized in the lung of bovine. In case of stressful conditions i.e. transportation, those viruses combined with other agents can lead to clinical respiratory disorders in cattle. Results of this study point out that IVM moderately inhibits replication of BRSV at in vitro conditions, compared to BPIV-3. It would be interesting to evaluate those results in clinical conditions. On the other hand, our results presents the first data on the inhibitory effect of IVM on pneumoviruses (*Pneumoviridae*), despite having some data on the Newcastle virus (*Paramyxoviridae*) (Azeem et al., 2015). Because of the close similarities of both viruses, it will also share possibilities for human respiratory syncytial virus research and treatments.

There are several reports about the inhibitory effects of IVM on varicelloviruses (*Herpesviridae*) including BoHV-1. The suggested mechanism of action is selective inhibition of UL42 DNA polymerase (Lv et al., 2018; Raza et al., 2020). For BoHV-1, Raza et al. (2020) suggested a fourfold decrease in viral titer, and ~44 % reduction in virion production by using of 25 μM IVM in MDBK cells. This level of treatment was found toxic for MDBK cultures in this study. But our results both in the experiment #1 and #4 were quite satisfying which represents a time depended increase in the efficiency that leads to at least fourfold decrease in viral titer at 120th h of treatment (Figs. 3 and 4). Similar results on pseudorabies virus (Lv et al., 2018) confirms the in vitro efficiency of IVM on various members of the genus *Varicellovirus*.

In conclusion, our findings demonstrated that IVM inhibits in vitro extracellular shedding of infectious virions and replication of BRSV, BPIV-3, BoHV-1, BCoV and BVDV in a dose-dependent manner. Although there is no result on demonstration of exact mechanism of action in this study, as a preliminary result, we suggest no effect on virus attachment and penetration into the host cell posed by IVM. Further clinical studies can help to clarify the possible therapeutic role of the
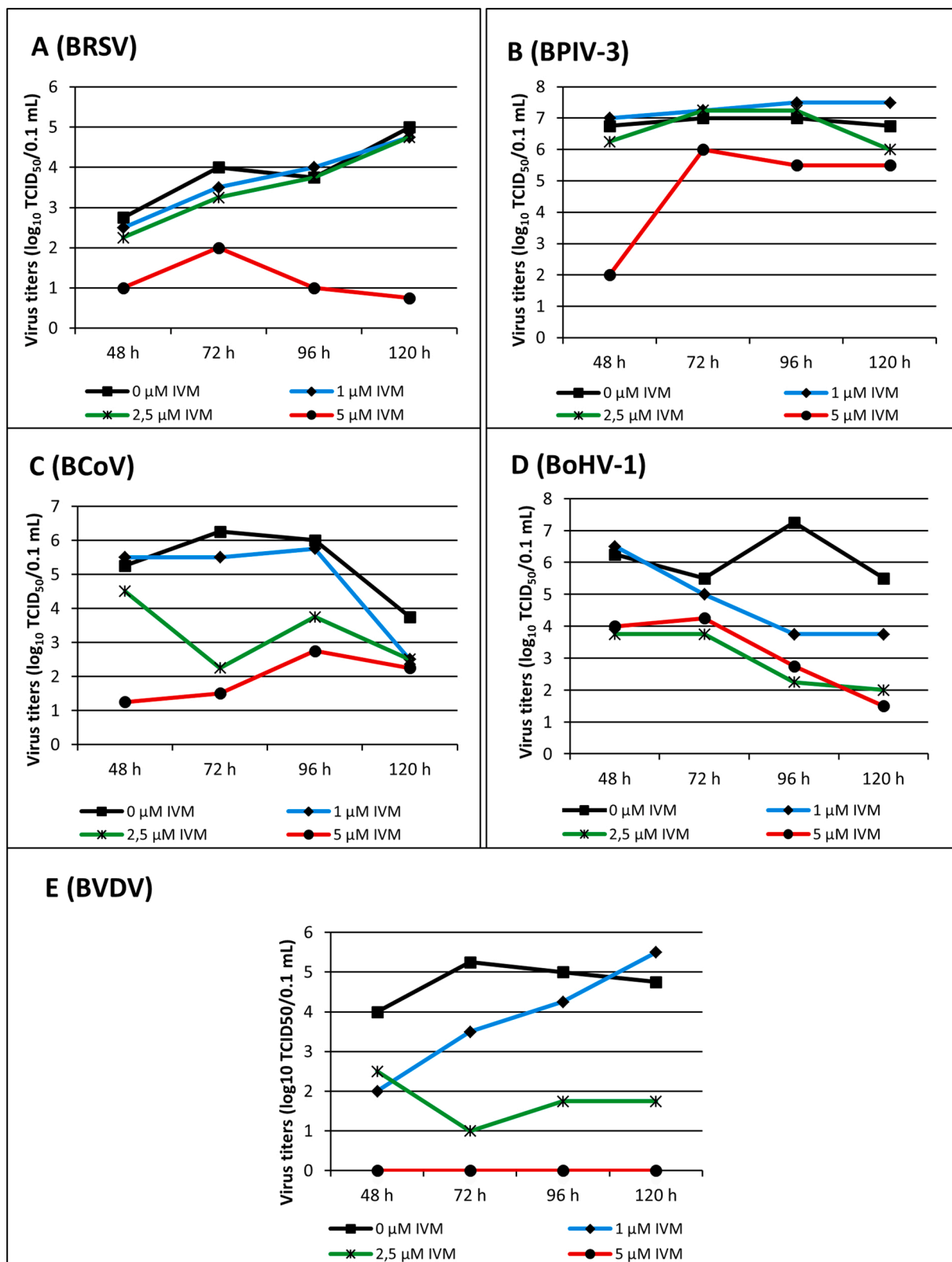
Fig. 4. Time dependent kinetics of infective viral particle counts in infected MDBK cell culture fluids treated with various ivermectin doses.
MDBK cell cultures infected with the viruses were cultured with DMEM containing 0, 1, 2.5 and 5 μM ivermectin. Culture fluids were collected from individual wells at defined periods with 24 h intervals. Detected virus titers are presented as Log_{10} $TCID_{50}$.

IVM.

Author statement

Kadir Yesilbag: Conceptualization, Methodology, Supervising, Writing, Redactions, Funding acquisition.

Eda Baldan Toker: Writing- Original draft preparation, Validation, Investigation, Formal analysis

Ozer Ates: Resource, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2021.198384.

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