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# DF-1-Derived exosomes mediate transmission of reticuloendotheliosis virus and resist REV-specific antibodies

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## Abstract

**Background** *Reticuloendotheliosis virus* (REV), a member of the family Retroviridae, is a hot area of research, and a previous study showed that exosomes purified from REV-positive semen were not blocked by REV-specific neutralizing antibodies and established productive infections.

**Methods** To further verify the infectivity of exosomes from REV-infected cells, we isolated and purified exosomes from REV-infected DF-1 cells and identified them using Western blot and a transmission electron microscope. We then inoculated 7-day-old embryonated eggs, 1-day-old chicks and 23-week-old hens with and without antibody treatment. REV was administered simultaneously as a control.

**Results** In the absence of antibodies, the results indicated that REV-exosomes and REV could infect chicks, resulting in viremia and viral shedding, compared with the infection caused by REV, REV-exosomes reduced the hatching rate and increased mortality after hatching, causing severe growth inhibition and immune organ damage in 1-day-old chicks; both REV and REV-exosomes also could infect hens, however, lead to transient infection. In the presence of antibodies, REV-exosomes were not blocked by REV-specific neutralizing antibodies and infected 7-day-old embryonated eggs. However, REV could not infect 1-day-old chicks and 23-week-old hens.

**Conclusion** In this study, we compared the infectious ability of REV-exosomes and REV, REV-exosomes could escape from REV-specific neutralizing antibodies in embryonated eggs, providing new insights into the immune escape mechanism of REV.

**Keywords** Reticuloendotheliosis virus, Exosome, Pathogenicity, Antibody neutralization, Immune escape

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## Background

Reticuloendotheliosis is a common neoplastic disease caused by infection with the reticuloendotheliosis virus (REV), which is the third type of tumor virus after the Marek's disease virus (MDV) and avian leukosis virus (ALV) [1]. During poultry production, co-infection with REV and other immunosuppressive viruses become more prevalent, and REV, as an exogenous virus, can contaminate live avian vaccines, which poses a significant threat to the poultry industry [2, 3].

The REV transmission route includes horizontal and vertical transmission, and REV has been detected in cock semen, which could infect offspring after artificial insemination with REV-positive semen [4]. In a previous study, we found that REV-positive semen-derived exosomes contained REV whole genome RNAs, established productive infections, and ignored antibody neutralization [5]. In recent years, exosomes have received increasing attention as an important pathway for mediating immune escape. Wang et al. verified that exosome-mediated porcine reproductive and respiratory syndrome virus (PRRSV) transmission is not completely blocked by specific neutralizing antibodies against PRRSV [6].

To further investigate the infectivity of exosomes from REV-infected cells and the ability of REV-escaped neutralizing antibodies, in this study, REV-exosomes and free REV-inoculated 7-day-old embryonated eggs, 1-day-old chicks, and 23-week-old hens with and without antibodies, compared pathogenicity and the ability of escaped antibodies, thus providing novel data on the mechanism of exosome-mediated REV-escaped immunity.

## Methods

### Cell culture and viruses

DF-1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA) with 10% heat-inactivated fetal calf serum (FBS; Gibco, Carlsbad, CA) and 1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO<sub>2</sub>. REV strain IBD-C1605 (GenBank accession number: KX278301) was isolated from a contaminated IBD vaccine [7]. In this study, DF-1 cells were passed and cultured overnight to 80% confluence, were infected with REV at a multiplicity of infection (MOI) of 1.0, after 3 passages of cells that were inoculated with REV, the culture supernatants were harvested and stored at -80°C, and the viral titer was measured by 50% median tissue culture infective dose (TCID<sub>50</sub>).

### Exosome isolation and purification

REV-exosomes or mock-infected DF-1 cell supernatants were collected and centrifuged for 5 min at 4°C to discard the cells and larger debris. The supernatant was transferred to a new tube and centrifuged at 2,000 × g for 20 min to remove cell debris. Then the supernatants

were centrifuged at 10,000 × g for 30 min and filtered through a 0.22-μm filter (Merck Millipore, USA). The filtrates were centrifuged at 10,000 × g for 90 min at 4°C, the products were collected and suspended in 50–500 μL of particle-free phosphate-buffered saline (PBS). Exosomes were purified according to a previously published method [6].

### Transmission electron microscopy (TEM) and detection of REV whole genome

The morphology of exosomes isolated from REV-infected DF-1 cell culture supernatant was evaluated using TEM (Hitachi H-7000FA, Japan). A drop (10 μL) of exosomes was placed on a carbon-coated copper grid (200 mesh), the exosomes had an adsorption period of 1 min at room temperature, and the excess liquid was absorbed by a clean filter paper. Then, the exosome-containing grids were incubated with 1% (w/v) uranyl acetate at room temperature for 1 min, and filter paper was utilized to absorb excess uranyl acetate. The copper mesh was placed under a transmission electron microscope for evacuation.

The total DNA was isolated from the purified exosomes using a cell genome DNA extraction kit (Omega Bio-Tek, USA), according to the manufacturer's protocol. PCR was performed with TakaRa Ex taq enzyme reagent kits (TakaRa Bio, Japan), following the manufacturer's instructions. The amplification products were detected by 1.0% agarose gel electrophoresis. Three pairs of primers used in this study according to a previously study [7].

### Western blot of exosomes and liquid chromatography-tandem mass spectrometry

Western blot was performed using the following established protocol, and total proteins were extracted from mock- or REV-exosomes using a modified RIPA buffer. The protein concentration was measured using a bicinchoninic acid assay (BCA) protein assay kit (NCM Biotech, China). Exosome lysates were subsequently loaded onto SDS-PAGE (4% stacking gel, 10% running gel), after electrophoresis, the resolved proteins were transferred onto 0.2-μm polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membrane was blocked with 5% BSA for 2 h at room temperature, then the membrane was incubated with the primary antibodies which were directed against CD63 and HSP70 (Abcam, UK) at 4°C overnight. The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, including goat anti-rabbit IgG (Beyotime Biotechnology, China). Antigen-antibody binding was visualized using enhanced chemiluminescence (ECL, Bio-Rad).

For MS analysis, Radio Immunoprecipitation Assay (RIPA) lysis and extraction buffer were added to the

purified exosomes, then centrifuged the sample at 12,000 rcf for 10 min, the supernatant was collected. Then proteins were digested with FASP procedure, after digestion, the peptide was desalted using a self-priming desalting column, and the solvent was evaporated in a vacuum centrifuge at 45°C. Experiments were performed on a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo, USA) coupled to an Easy-nLC 1200 liquid chromatograph (Thermo, USA).

#### Establishment and selection of REV antibody positive in embryonated eggs and chickens

Formaldehyde-inactivated REV was emulsified with an oil adjunct to produce a REV oil emulsion-inactivated vaccine. The immunization was carried out thrice 8 weeks before the first laying of SPF hens; blood samples were collected weekly after the third immunization, REV-specific antibodies were detected by Enzyme-linked immunosorbent assay (IDEXX) kit, hens with positive REV antibodies were picked, their embryonated eggs were collected, the extracted egg yolks were detected using an ELISA kit, and antibody-positive embryonated eggs were stored. A portion of embryonated eggs with REV antibody-positive hatched blood samples from 1-day-old chicks was collected to detect maternal antibodies, and chicks with positive REV antibodies were used in the later experiments.

#### Infectivity of REV and REV-exosome in embryonated eggs

To further compare the infectivity of different infections, REV exosomes or free REV at the same TCID<sub>50</sub> (500) were used to infect 7-day-old embryonated eggs with and without antibodies. Blood and cloacal swabs were collected at 1, 7, 14, and 21 day post infection after

hatching, and REV was detected by quantitative real-time PCR. The total RNAs of samples were extracted with the E.Z.N.A total RNA kit I (Omega Bio-tek), the isolated RNA was reverse transcribed into cDNA using M-MLV RT Kit (TakaRa) per the manufacturer's protocol, quantitative real-time PCR was performed with SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology, China), the primers used to quantify the REV were as follows: 5'-CCCCATTCATGTCCAGCTAT-3' and 5'-AGGGAGGA GAGGAGTGTTC-3'.

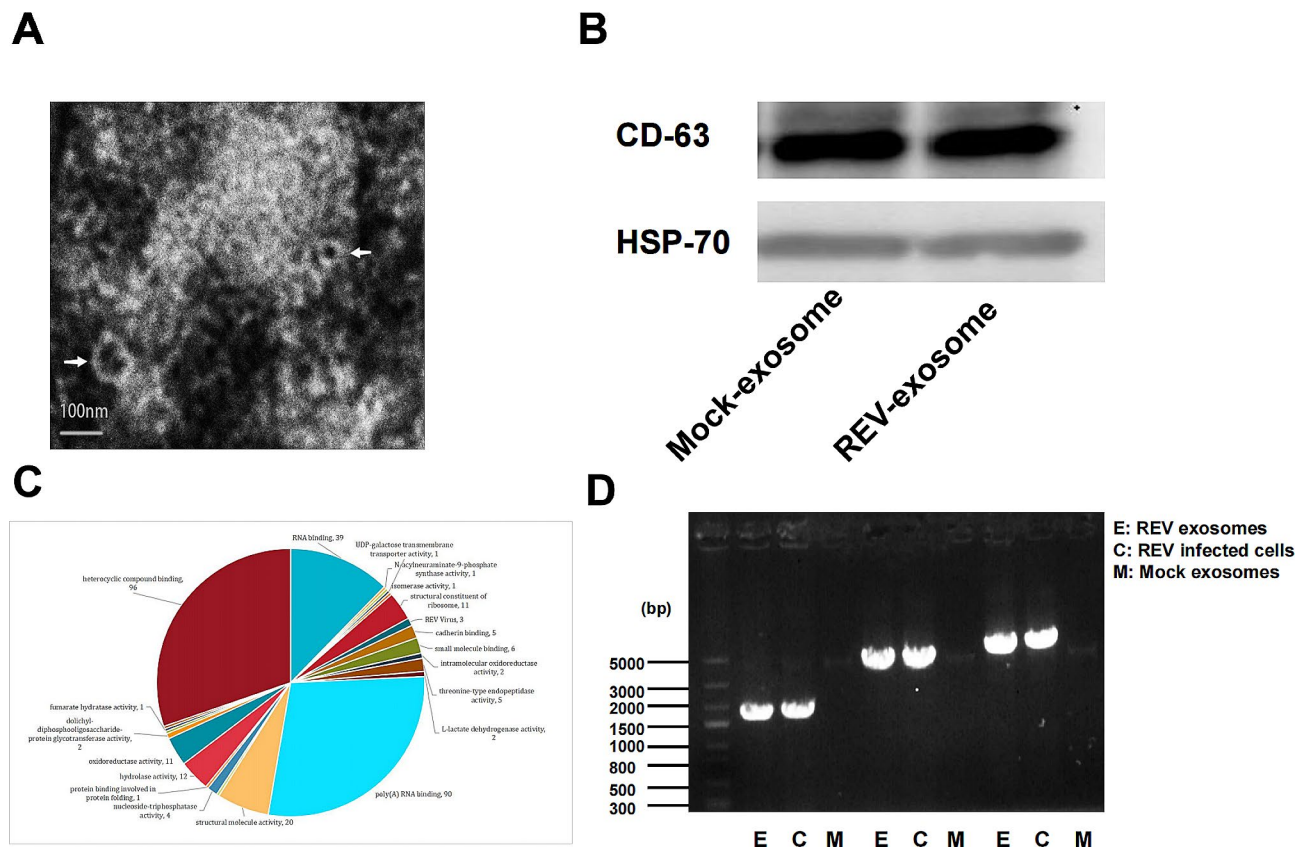
#### Infectivity of REV and REV-exosome in chickens

REV-exosome or free REV at the same TCID<sub>50</sub> (1000) were used to infect 1-day-old chicks with and without antibodies, blood, cloacal swabs, and immune organs were collected at 7, 14 and 21 dpi, the spleen, bursa, and thymus were weighted and stored, the mRNA levels of IFN-α, IFN-β, IFN-γ and Mx genes in liver and spleen were detected and analyzed. Primers used for real-time polymerase chain reaction (PCR) shown in Table 1. The SPSS version 25 was used for statistical analysis. The relative quantification of gene expression was normalized by normalization to the level of β-actin, and the expression levels of genes in the REV-infected group were arbitrarily set to 1. Utilized the 2<sup>-ΔΔCt</sup> method to analyze the relative RNA expression levels. Data were analyzed and plotted using the GraphPad Prism 6 software.

REV-exosomes or free REV at the same TCID<sub>50</sub> (10,000) were used to infect 23-week-old hens with and without antibodies, blood and cloacal swabs were collected at 7, 14, 21, 28, and 35 dpi, and REV was detected by quantitative real-time PCR.

**Table 1** Primers used for real-time PCR.

	Primer sequence	GenBank accession no.	Product size (bp)
REV-1	F: 5'-AATGTGGGAGGGAGCTCTGGGGGA-3' R: 5'-CCTGATCATTCTGACCTCCCGCC-3'	KX278301	1798
REV-2	F: 5'-ACCAGACTCTACTGTAATGGCGAGCC-3' R: 5'-AATACTGAGGGTTTCGGTATCTGG-3'	KX278301	3401
REV-3	F: 5'-TCTGGCTACCTAAACGGGTAGCTGT-3' R: 5'-CCCCAAATGTTGTACCGAAATACTA-3'	KX278301	3660
IFN-α	F: 5'-CGCAACCTTCACCTCACCATCAAA-3' R: 5'-TGTGAGGTTGTGGATGTGCAGGAA-3'	NM-205427.1	93
IFN-β	F: 5'-GCCCACACACTCCAAAACACTG-3' R: 5'-TTGATGCTGAGGTGAGCGTTG-3'	KF-741874.1	151
IFN-γ	F: 5'-AAGTCAAAGCCGCACATCAAAC-3' R: 5'-CTGGATTCTCAAGTCGTTTCATCG-3'	NM-205149.1	132
Mx	F: 5'-CAGCTCCAGAATGCATCAGA-3' R: 5'-GGCAATCCAGGAAGATCAA-3'	XM-046906529.1	156
β-actin	F: 5'-GAGAAATTGTGCGTGACATCA-3' R: 5'-CCTGAACCTCTCATTGCCA-3'	NM-205518.2	152



**Fig. 1** Isolation and characterization of exosomes from REV-infected DF-1 cells. **(A)** Transmission electron microscopy observation of negatively stained the exosome, scale bar = 100 nm. **(B)** Purified exosomes derived from mock- or REV-infected DF-1 cells were analyzed on Western blot with antibodies against CD63 and HSP70. **(C)** LC-MS/MS analysis of the purified exosomes from REV-infected DF-1 cells, to detect the presence of REV proteins in exosomes. **(D)** Three pairs of primers designed against REV genome sequence in purified exosomes isolated from REV-infected DF-1 cells, the targeted fragments were amplified with sizes of 1798, 3401 and 3632 bp

**Results**

**Isolation, identification, and component analysis of REV-infected DF-1 cells**

The cell supernatant was collected 24 h after REV-infected DF-1 cells, and exosomes were isolated through differential centrifugation, TEM demonstrated that exosomes displayed a cup-shaped appearance ranging from about 100 nm in size (Fig. 1A), which conformed to the size and morphology of exosomes, Western blot was used to analyze the exosomal markers, two representative exosome markers, CD63 and HSP70 were detected (Fig. 1B). These results indicated that the exosomes were successfully isolated. Furthermore, liquid chromatography-tandem mass spectrometry (LC-MS/MS) found the three REV-related proteins, namely, pol-gag polyprotein, env and REV-polymerase (Fig. 1C). The whole-genome of REV was detected in the purified exosomes from REV-infected DF-1 cells (Fig. 1D).

**Lethality of REV exosomes and REV on embryonated eggs**

Fifty embryonated eggs were divided into two groups: 10 embryonated eggs hatched in the REV-exosome group,

**Table 2** Hatching rate of 7-day-old embryonated eggs infected with REV-exosome or REV

Groups	embryonated eggs without antibodies	embryonated eggs with antibodies
REV-exosome group	40.0% (10/25)	36.0% (9/25)
REV group	68.0% (17/25)	84.0% (21/25)

with a hatching rate of 40%, and 17 embryonated eggs hatched in the REV group, with a hatching rate of 68% (Table 2). REV-exosomes significantly reduced the hatching rate ( $P < 0.05$ ). Fifty embryonated eggs with antibodies were divided into two groups: 9 embryonated eggs hatched in the REV-exosome group, with a hatching rate of 36%, and 21 embryonated eggs hatched in the REV group, with a hatching rate of 84% (Table 2). REV was detected in all dead embryonated eggs. The results showed that REV could be blocked by REV-specific neutralizing antibodies, REV-exosomes could infect embryonated eggs and resist neutralizing antibodies.

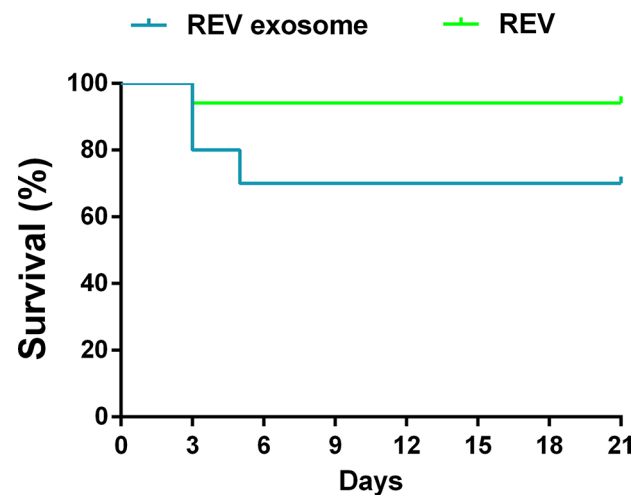
### Inoculation of REV-exosomes into 7-day-old embryonated eggs resulted in viremia and viral shedding

7-day-old embryonated eggs without antibodies were infected with REV or REV-exosome, and after hatching, the REV group died a chick at 3 d, REV-exosome group died 2 chickens at 3 d, died a chick at 5 d (Fig. 2), all dead chicks detected REV through qPCR. REV and REV-exosomes did not cause death after embryonated eggs hatched with antibodies.

REV-exosomes or free REV were used to infect 7-day-old embryonated eggs without antibodies and viremia was detected at 1, 7, 14, and 21 dpi; the positive rate of the REV-exosome group was higher than that of the REV group, and the REV-exosome group maintained a high positive rate for a long time (Table 3), suggesting that the REV-exosome group had stronger infectivity than in the REV group, and viral shedding was detected in the cloacal swab of the REV and REV-exosome groups (Table 3). We infected 7-day-old embryonated eggs with antibodies with REV or REV-exosomes, and viremia was detected at 1, 7, 14, and 21 dpi. In the presence of antibodies, REV-exosomes also infected embryonated eggs and maintained a high positive rate (Table 4). Simultaneously, cloacal swabs were also tested (Table 4). These results suggest that exosome-mediated REV infection could not be blocked by REV-specific neutralizing antibodies. However, REV did not infect embryonated eggs with antibodies, and viral shedding was not found in the REV group.

### Infectivity and pathogenicity of REV-exosome and REV in 1-day-old chicks

REV- or REV-exosome-infected 1-day-old chicks without antibodies and detection of viremia at 7, 14, and 21 dpi showed no differences in the early stages of infection. However, with an increasing number of days, the REV-exosome group was all infected, but the positive rate of the REV group decreased (Table 5). REV-exosomes and REV resulted in viral shedding as detected by cloacal swabs (Table 5). REV- and REV-exosome-infected 1-day-old chicks with antibodies, viremia, or viral shedding were not detected. Compared to the REV group, the REV-exosome group resulted in more severe growth inhibition and immune organ damage in chicks. The body weight of REV group (Fig. 3A) was significantly higher than that of REV-exosome group at 21 dpi ( $P < 0.05$ ), the spleen index



**Fig. 2** Seven-day-old embryonated eggs were infected with REV-exosomes or REV after hatching, and the chick survival rate was determined

**Table 3** Detection of viremia and cloacal swabs from embryonated eggs without antibodies

Time (days)	Positive rate of REV in blood		Positive rate of REV in cloacal swabs	
	REV-exosome	REV	REV-exosome	REV
1 d	80.0% (8/10)	58.8% (10/17)	80.0 (8/10)	23.5% (4/17)
7 d	85.7% (6/7)	56.3% (9/16)	85.7% (6/7)	43.8% (7/16)
14 d	100% (7/7)	56.3% (9/16)	57.1% (4/7)	62.5% (10/16)
21 d	100% (7/7)	37.5% (6/16)	28.6% (2/7)	100% (16/16)

**Table 5** Detection of viremia and cloacal swabs from 1-day-old chicks without antibodies

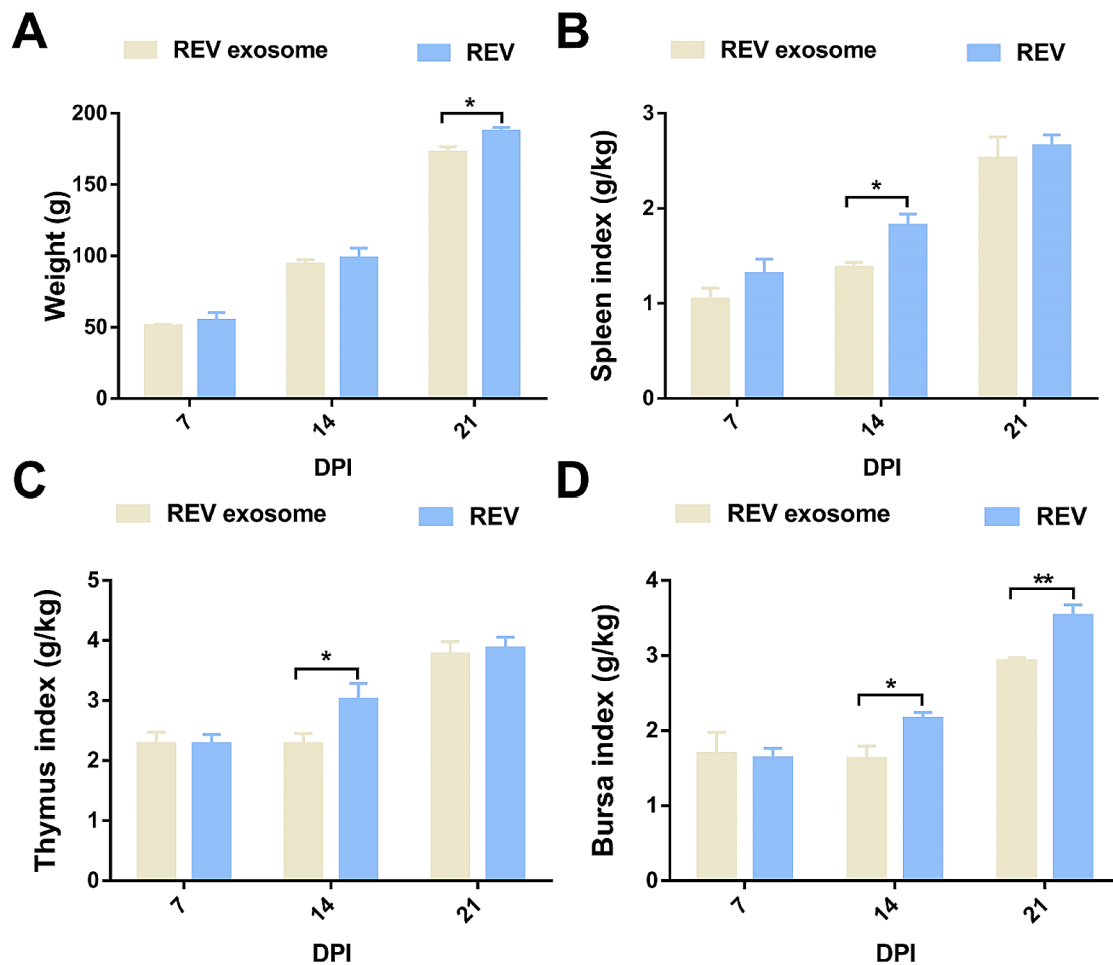
Time (days)	Positive rate of REV in blood		Positive rate of REV in cloacal swabs	
	REV-exosome	REV	REV-exosome	REV
7 d	80.0%(12/15)	80.0%(12/15)	80.0%(12/15)	80.0%(12/15)
14 d	100%(12/12)	100%(12/12)	83.3%(10/12)	41.7%(5/12)
21 d	100%(9/9)	55.6%(5/9)	55.6%(5/9)	77.8%(7/9)

(Fig. 3B) of REV-exosome group was significantly lower than group REV ( $P < 0.05$ ), the thymus index (Fig. 3C) of REV-exosome group was significantly lower than REV group at 14 dpi ( $P < 0.05$ ), and the bursa index (Fig. 3D) of REV-exosome group was significantly lower than REV group at 14 and 21 dpi ( $P < 0.05$ ,  $P < 0.01$ ). These results

**Table 4** Detection of viremia and cloacal swabs from embryonated eggs with antibodies

Time (days)	Positive rate of REV in blood		Positive rate of REV in cloacal swabs	
	REV-exosome	REV	REV-exosome	REV
1 d	33.3%(3/9)	0%(0/21)	33.3%(3/9)	0%(0/21)
7 d	66.7%(6/9)	0%(0/21)	66.7%(6/9)	0%(0/21)
14 d	66.7%(6/9)	0%(0/21)	66.7%(6/9)	19.0%(4/21)
21d	66.7%(6/9)	0%(0/21)	33.3%(3/9)	0%(0/21)





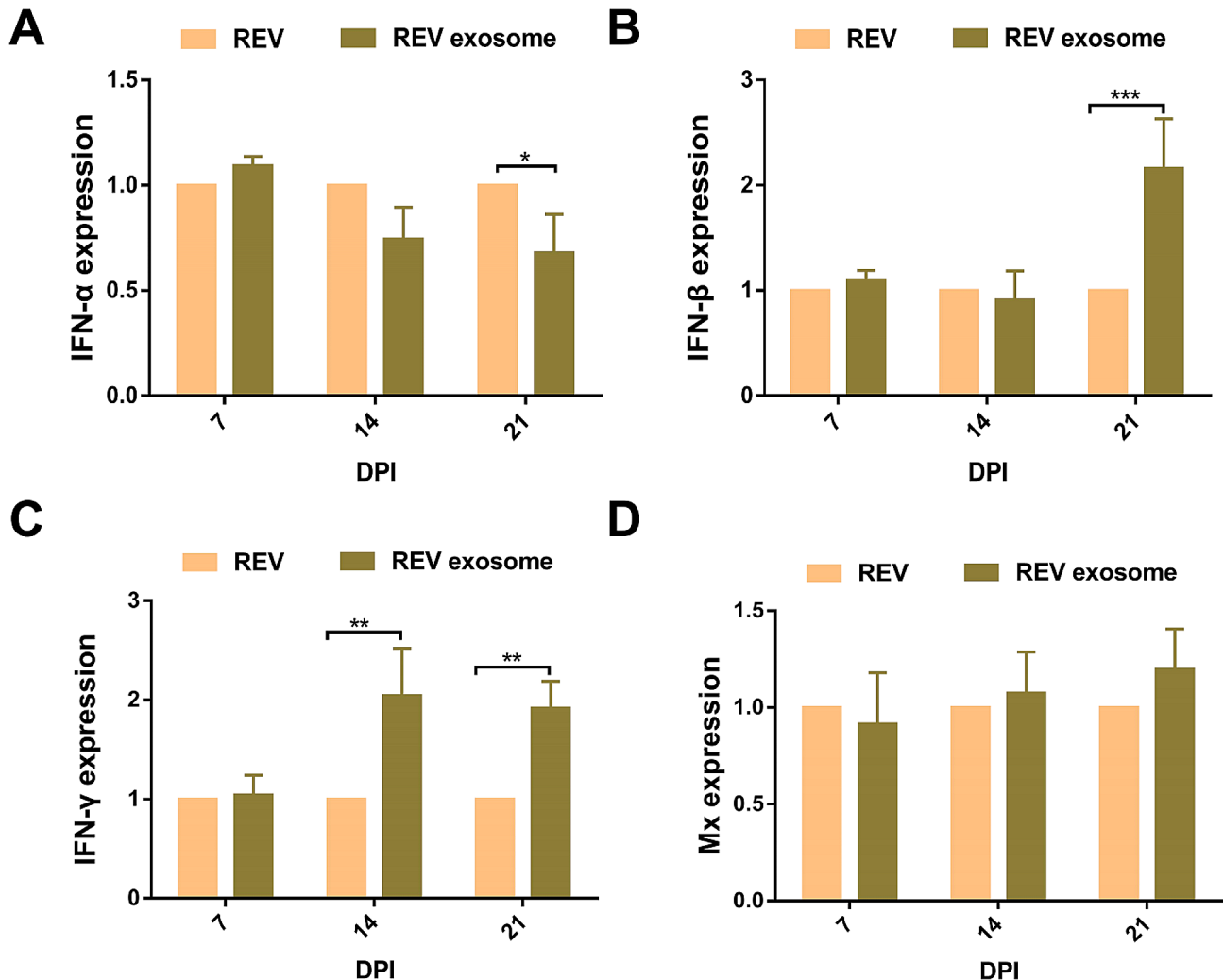
**Fig. 3** One-day-old chicks were infected with REV-exosomes or REV, and the body weight and immune organ index were recorded. The body weights of the infected chicks were recorded at 7, 14, and 21 dpi. The whole spleen, thymus, and bursa from the infected chicks were collected at 7, 14, and 21 dpi, weighed, and recorded. The body weights of the infected chicks were recorded at 7, 14, and 21 dpi. (A) Changes in body weight from 1 to 21 dpi. (B–D) Changes in immune organ index from 1 to 21 dpi. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

indicated that REV-exosome induced more severe atrophy of the thymus, spleen, and bursa. In 1-day-old chicks with antibodies, no differences were observed between the REV-exosome and REV groups.

#### Detection of immune-related genes after REV-exosome or REV-infected 1-day-old chicks

REV or REV-exosome infected 1-day-old chicks without antibodies and their liver and spleen samples collected at 7, 14, and 21 dpi, and the IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , and Mx mRNA levels were detected using qRT-PCR. In the spleen, the IFN- $\alpha$  mRNA level (Fig. 4A) in the REV-exosome group was significantly lower than this in the REV group at 21 dpi ( $P < 0.05$ ), the IFN- $\beta$  mRNA level (Fig. 4B) in the REV-exosome group was significantly higher at 21 dpi ( $P < 0.001$ ), and the IFN- $\gamma$  mRNA levels (Fig. 4C) in REV-exosome group was significantly higher at 14 and 21 dpi ( $P < 0.01$ ). However, there was no difference in the

Mx mRNA levels (Fig. 4D) between the REV-exosome group and the REV group. At the same time, we plot the expression levels of both groups over time in the spleen, each group used the cytokine mRNA levels on day 7 as a control, the IFN- $\alpha$  mRNA levels of REV-exosome group (Fig S4A) was significantly decreased at 21 dpi ( $P < 0.05$ ). The IFN- $\alpha$  and IFN- $\beta$  mRNA levels of REV group (Fig S4E and F) were also significantly decreases at 21 dpi ( $P < 0.05$ ,  $P < 0.01$ ). In the liver, the IFN- $\alpha$  (Fig. 5A) and Mx (Fig. 5D) mRNA levels were not significantly different, the IFN- $\beta$  (Fig. 5B) mRNA level in the REV-exosome group was significantly higher than this of the REV group at 14 dpi ( $P < 0.01$ ), the IFN- $\gamma$  (Fig. 5C) transcription level was significantly higher at 21 dpi ( $P < 0.001$ ). We also plot the expression levels of both groups over time in the liver, each group used the cytokine mRNA levels on day 7 as a control, the IFN- $\alpha$ , IFN- $\beta$  and IFN- $\gamma$  mRNA levels (Fig S5A, B and C) were significantly upregulated in



**Fig. 4** mRNA levels of immune-related genes in the spleens of infected chicks. 1-day-old chicks were infected with REV-exosome or REV, and spleen samples were collected at 7, 14, and 21 dpi. The immune-related genes in the spleens were detected by real-time quantitative reverse transcription polymerase chain reaction analysis, and relative expression levels were normalized to the  $\beta$ -actin gene and calculated using the  $2^{-\Delta\Delta Ct}$  method. (A–D) IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$  and Mx expression levels. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

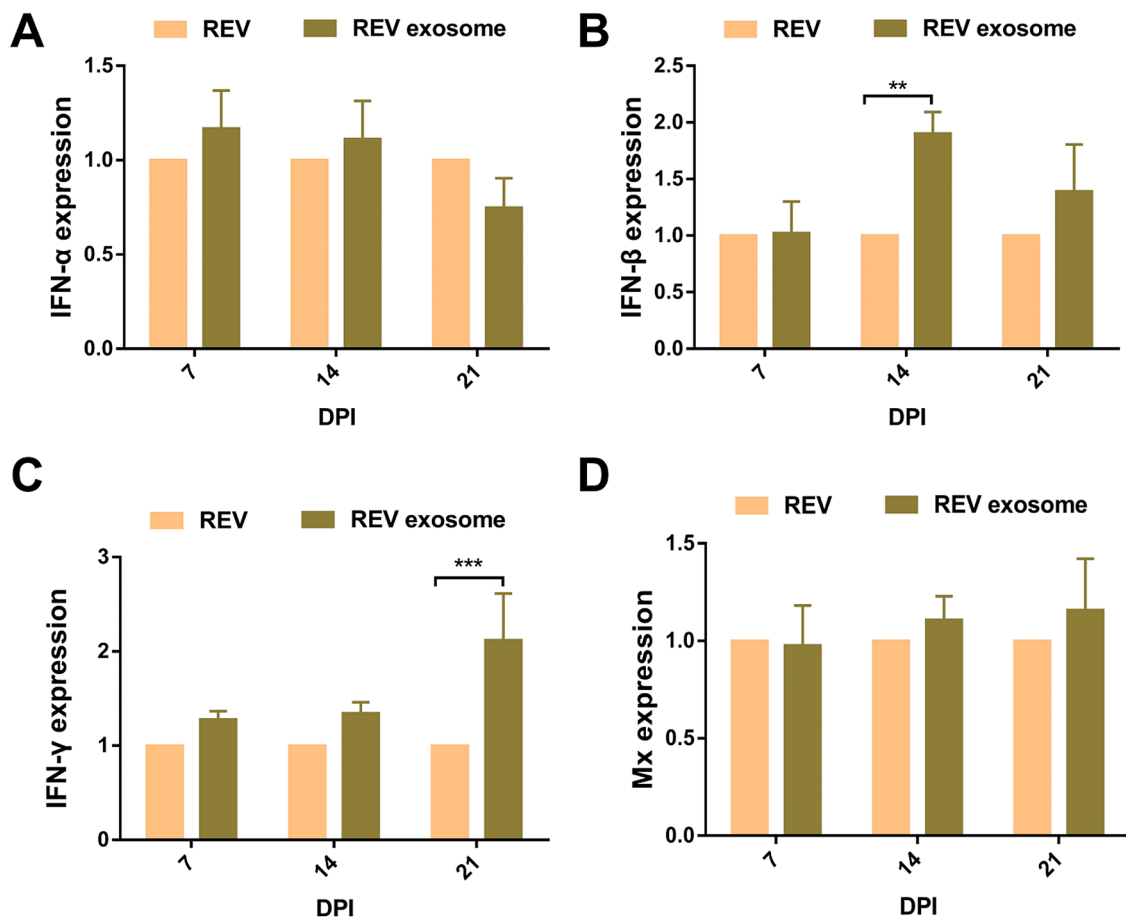
REV-exosome group at 14 dpi ( $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.05$ ), IFN- $\alpha$  and IFN- $\beta$  mRNA levels (Fig S5A and B) were significantly decreased at 21 dpi ( $P < 0.01$ ,  $P < 0.05$ ). In REV group, the IFN- $\alpha$  and IFN- $\gamma$  mRNA levels (Fig S5E and G) were significantly upregulated ( $P < 0.01$ ,  $P < 0.05$ ), the IFN- $\alpha$ , IFN- $\gamma$  and Mx mRNA levels (Fig S5E, F and H) were significantly decreased ( $P < 0.01$ ,  $P < 0.01$ ,  $P < 0.01$ ). The results suggested that the REV-exosome-induced immune response was stronger than that of REV. In the early stage of infection, exosome-mediated REV infection was not recognized by the immune system, which facilitated the replication and proliferation of viruses, whereas in the later stage of infection, massive proliferation of the virus resulted in strong immune responses. Through plotting the expression levels of both groups over time, we found that two groups showed similar trends in cytokine changes, which may be related to viral proliferation.

#### Susceptibility of REV-exosome and REV in 23-week-old hens

REV-exosome and REV infected 23-week-old hens without antibodies, viremia was detected at 7, 14, and 21 dpi, and both REV-exosome and REV could infect hens for a period of time (Table 6), leading to transient infection. Viral shedding was not detected in the cloacal swabs (Table 6). REV-exosome and REV-infected 23-week-old hens with antibodies, viremia, or viral shedding were not detected.

#### Discussion

Even if the host immune system makes a response and produces neutralizing antibodies, certain viruses can hijack secretory pathways in extracellular vesicles to escape antibody recognition, Johnstone et al. named these vesicles “exosomes” [8], the exosomes play



**Fig. 5** mRNA levels of immune-related genes in the livers of infected chicks. 1-day-old chicks were infected with REV-exosome or REV, and liver samples were collected at 7, 14, and 21 dpi. The immune-related genes in the livers were detected by real-time quantitative reverse transcription polymerase chain reaction analysis, and relative expression levels were normalized to the  $\beta$ -actin gene and calculated using the  $2^{-\Delta\Delta C_t}$  method. (A–D) IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$  and Mx expression levels. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

**Table 6** Detection of viremia and cloacal swabs from 23-week-old hens without antibodies

Time (days)	Positive rate of REV in blood		Positive rate of REV in cloacal swabs	
	REV-exosome	REV	REV-exosome	REV
7 d	0%(0/15)	0%(0/15)	0%(0/15)	0%(0/15)
14 d	60%(9/15)	40%(6/15)	0%(0/15)	0%(0/15)
21 d	0%(0/15)	0%(0/15)	0%(0/15)	0%(0/15)

important roles in intercellular communication and signal transduction [9–11]. Retrovirus utilized formation and secretory pathways of exosomes in host cells to produce infectious virus particles, productive infection was established by receptors independent pathway [12]. Exosomes derived from hepatitis C virus (HCV)-infected cells carry viral RNA and can mediate the viral-receptor-independent transmission of HCV [13]. Exosomes isolated from human immunodeficiency virus (HIV)-infected cells make the recipient cells more susceptible to HIV infection [14]. In recent years, exosomes

have received more attention as an important pathway for mediating immune escape, and our previous studies indicated that REV-positive semen-derived exosomes not only included REV whole-genome RNAs but also established productive infection and escaped neutralizing antibodies [5]. Exosomes from CEF infected with ALV-J or REV contained various viral genes and structural proteins [15]. This study aimed to determine whether exosomes from cells infected with REV establish productive infections in embryonated eggs, chicks, and hens and whether exosomes establish productive infection by evading the clearance of neutralizing antibodies.

In this study, we found that REV utilized exosomes to establish productive infections and has stronger pathogenicity. To determine exosome infectivity, we first utilized the extracted exosomes to infect 7-day-old embryonated eggs, 1-day-old chicks, and 23-week-old hens, which confirmed that exosomes from cells infected with REV resulted in the infection of embryonated eggs and chicks, which had strong infectivity. Why does REV-exosome



group had strong infectivity? We think that exosomes are not easily recognized by the immune system, in the early stages of viral infection, REV-exosome evade the body's immune surveillance, therefore, REV can more effectively replicate and proliferate. Compared to REV, REV-exosome has stronger pathogenicity.

When REV faced the blocking effect of REV-specific neutralizing antibodies, we investigated whether exosomes from cells infected with REV established productive infection in embryonated eggs, chicks, and hens and whether they established productive infection by evading the clearance of neutralizing antibodies. As an important pathway for immune escape, exosome-mediated viral infection cannot be blocked by specific neutralizing antibodies [6]. In this study, we found that REV-exosomes could infect embryonated eggs with antibodies, but REV was blocked by specific neutralizing antibodies and therefore could not infect embryonated eggs. The exosomes are a type of extracellular vesicle, many studies have shown that viruses could utilize EVs for cell-to-cell transmission [16–22], this strategy can help many virus to evade host immune surveillance and antibody neutralization. Gao et al. proved that apoptotic bodies (ApoBDs)-mediated viral transmission is fully resistant to swine sera to ASFV [23]. Our research also proved that REV-exosomes resist to antibody neutralization.

Compared to the REV group, the IFN- $\beta$  and IFN- $\gamma$  mRNA levels were significantly increased in the liver and spleen of the REV-exosome group, but in the early stage of infection, there were no differences in the immune responses between the REV-exosome group and the REV group. We hypothesized that REV-exosomes evade immune system surveillance in the early stages of infection, facilitating viral replication and proliferation. This massive viral proliferation induces a strong immune response and elevated cytokine levels. We plot the expression levels of both groups over time in the spleen and liver, the two groups were increased earlier and decreased later, showing the same trend, this may be related to proliferation of the virus.

## Conclusion

This study confirmed that REV-exosomes could establish a productive infection in embryonated eggs; 1-day-old chicks and 23-week-old hens had a stronger pathogenicity than REV. Exosome-mediated REV infection was not blocked by REV-specific neutralizing antibodies; thus, 7-day-old embryonated eggs were infected with REV and chicks carried REV for a long time. This study further enriches the data on exosomes enhancing the REV infectivity and provides novel insights into the REV immune escape mechanism.

## Abbreviations

REV	Reticuloendotheliosis virus
MDV	Marek's disease virus
ALV	Avian leukosis virus
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal calf serum
IBD	Infectious bursal disease
TCID50	50% median tissue culture infective dose
TEM	Transmission electron microscope
PVDF	Polyvinylidene difluoride
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
CEF	Chicken embryo fibroblasts

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12985-024-02445-4>.

Supplementary Material 1: **Figure supplement 1.** Transmission electron microscopy observation of negatively stained the exosome.

Supplementary Material 2: The western blot with antibody against CD63.

Supplementary Material 3: The western blot with antibody against HSP70.

Supplementary Material 4: **Figure supplement 4.** mRNA levels of immune-related in the spleen. Each group used the cytokine mRNA levels on day 7 as a control, relative expression levels were normalized to the  $\beta$ -actin gene and calculated using the  $2^{-\Delta\Delta Ct}$  method. (A-D) IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$  and Mx expression levels of REV-exosome group. (E-H) IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$  and Mx expression levels of REV group. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

Supplementary Material 5: **Figure supplement 5.** mRNA levels of immune-related in the liver. Each group used the cytokine mRNA levels on day 7 as a control, relative expression levels were normalized to the  $\beta$ -actin gene and calculated using the  $2^{-\Delta\Delta Ct}$  method. (A-D) IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$  and Mx expression levels of REV-exosome group. (E-H) IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$  and Mx expression levels of REV group. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

## Author contributions

WZ conceived and performed the experiments, analyzed the data, and drafted the manuscript. PZ supervises the project and edited the manuscript. HZC and YWZ contributed to the experimental techniques and materials, WLS and WJY helped to analyze the data. All authors contributed to the article and approved the submitted version.

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## Data availability

No datasets were generated or analysed during the current study.

## Declarations

### Ethics approval and consent to participate

The animal care and use protocol were approved by the Shandong Agricultural University Animal Care and Use Committee (SDAUA-2016-002). All the experimental animals of this study were cared for and maintained throughout the experiments strictly following the ethics and biosecurity guidelines approved by the Institutional Animal Care and Use Committee of Shandong Agricultural University.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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## References

1. Campbell WF, Baxter-Gabbard KL, Levine AS. Avian reticuloendotheliosis virus (strain T). I. Virological characterization. *Avian Dis.* 1971;15(4):837–49.
2. Li J, Dong X, Yang C, Li Q, Cui Z, Chang S, et al. Isolation, identification, and whole genome sequencing of reticuloendotheliosis virus from a vaccine against Marek's disease. *Poult Sci.* 2015;94(4):643–9.
3. Wei K, Sun Z, Zhu S, Guo W, Sheng P, Wang Z, et al. Probable congenital transmission of reticuloendotheliosis virus caused by vaccination with contaminated vaccines. *PLoS ONE.* 2012;7(8):e43422.
4. Su Q, Li Y, Cui Z, Chang S, Zhao P. The experimental transmission of reticuloendotheliosis virus by cock semen. *J Gen Virol.* 2018;99(2):253–7.
5. Su Q, Zhang Y, Cui Z, Chang S, Zhao P. Semen-derived Exosomes Mediate Immune escape and transmission of Reticuloendotheliosis Virus. *Front Immunol.* 2021;12:735280.
6. Wang T, Fang L, Zhao F, Wang D, Xiao S. Exosomes mediate intercellular transmission of Porcine Reproductive and Respiratory Syndrome Virus. *J Virol.* 2018;92(4).
7. Li Y, Cui S, Cui Z, Chang S, Zhao P. Genome analysis and pathogenicity of reticuloendotheliosis virus isolated from a contaminated vaccine seed against infectious bursal disease virus: first report in China. *J Gen Virol.* 2016;97(11):2809–15.
8. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem.* 1987;262(19):9412–20.
9. Théry C, Duban L, Segura E, Véron P, Lantz O, Amigorena S. Indirect activation of naïve CD4+T cells by dendritic cell-derived exosomes. *Nat Immunol.* 2002;3(12):1156–62.
10. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol.* 2007;9(6):654–9.
11. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche I, Sena-Estevés M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol.* 2008;10(12):1470–6.
12. Gould SJ, Booth AM, Hildreth JE. The Trojan exosome hypothesis. *Proc Natl Acad Sci U S A.* 2003;100(19):10592–7.
13. Bukong TN, Momen-Heravi F, Kodys K, Bala S, Szabo G. Exosomes from Hepatitis C infected patients transmit HCV infection and contain replication competent viral RNA in Complex with Ago2-Mir122-HSP90. *PLoS Pathog.* 2014;10(10):e1004424.
14. Narayanan A, Iordanskiy S, Das R, Van Duyne R, Santos S, Jaworski E, et al. Exosomes derived from HIV-1-infected cells contain transactivation response element RNA. *J Biol Chem.* 2013;288(27):20014–33.
15. Zhou D, Xue J, He S, Du X, Zhou J, Li C, et al. Reticuloendotheliosis virus and avian leukosis virus subgroup J synergistically increase the accumulation of exosomal miRNAs. *Retrovirology.* 2018;15(1):45.
16. Escribano JM, Galindo I, Alonso C. Antibody-mediated neutralization of African swine fever virus: myths and facts. *Virus Res.* 2013;173(1):101–9.
17. Urbanelli L, Buratta S, Tancini B, Sagini K, Delo F, Porcellati S et al. The role of Extracellular vesicles in viral infection and Transmission. *Vaccines (Basel).* 2019;7(3):102.
18. Zeng C, Evans JP, King T, Zheng YM, Oltz EM, Whelan SPJ, et al. SARS-CoV-2 spreads through cell-to-cell transmission. *Proc Natl Acad Sci U S A.* 2022;119(1):e2111400119.
19. Chen YH, Du W, Hagemeyer MC, Takvorian PM, Pau C, Cali A, Brantner CA, Stempinski ES, Connelly PS, Ma HC, Jiang P, Wimmer E, Altan-Bonnet G, Altan-Bonnet N. Phosphatidylserine vesicles enable efficient en bloc transmission of enteroviruses. *Cell.* 2015;160(4):619–30.
20. Mercer J, Helenius A. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science.* 2008;320(5875):531–5.
21. Morris-Love J, Gee GV, O'Hara BA, Assetta B, Atkinson AL, Dugan AS, Haley SA, Atwood WJ. JC Polyomavirus uses extracellular vesicles to infect Target cells. *mBio.* 2019;10(2):e00379–19.
22. Ramakrishnaiah V, Thumann C, Fofana I, Habersetzer F, Pan Q, de Ruiter PE, et al. Exosome-mediated transmission of hepatitis C virus between human hepatoma Huh7.5 cells. *Proc Natl Acad Sci U S A.* 2013;110(32):13109–13.
23. Gao P, Zhou L, Wu J, Weng W, Wang H, Ye M, et al. Riding apoptotic bodies for cell-cell transmission by African swine fever virus. *Proc Natl Acad Sci U S A.* 2023;120(48):e2309506120.

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