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Chicken egg yolk antibodies against bovine respiratory syncytial virus neutralize the virus *in vitro*.

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Abstract

Bovine respiratory syncytial virus (BRSV) and its counterpart in humans (HRSV) are two closely related virus, which are the leading cause of severe respiratory syndrome in calves and young children, respectively. Passive immunization can be a practical alternative to conventional vaccination in order to prevent the disease. In this report the production of chicken egg yolk IgY and its ability to neutralize BRSV *in vitro* were assessed. Purified IgY against BRSV specifically recognized BRSV in a dot blot assay and was able to neutralize the virus in a viral neutralization assay. These results demonstrate the potential use of IgY as a prophylactic treatment against RSV infection.

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Keywords: bovine respiratory syncytial virus; respiratory disease; egg yolk antibodies; IgY technology; passive protection.

1. Introduction

Bovine respiratory syncytial virus (BRSV) is an enveloped, non-segmented, negative-stranded RNA virus and it is the main cause of respiratory disease in young calves [1]. BRSV is closely related to human respiratory syncytial virus (HRSV) which affects young children causing severe pneumonia. The epidemiology and pathogenesis of infection of both viruses are quite similar [2]. These features make BRSV infection in calves a good model for the study of HRSV [3].

BRSV affects cattle of all ages, especially calves from three to twelve months, causing respiratory tract infections that can reach 100% morbidity and 5-20% mortality. There are many vaccines available in the market, either inactivated or live attenuated, most of them as combination vaccines with other antigens related to the bovine respiratory disease complex [4]. However, there is a growing demand about the development of new generation vaccines, which become more effective, even in the presence of maternal antibodies and capable of inducing long-lasting protection over time. An interesting alternative to the use of conventional vaccines for the prevention of respiratory disease caused by BRSV is the administration of antibodies as passive therapy.

Antibodies play a major role in protection against RSV. Although infection occurs even in the presence of neutralizing antibodies, they are considered to be protective as high titers decrease the severity of the disease in both children and calves [5]. For this reason, it is important to develop a strategy to obtain protective antibodies against BRSV for subsequent use for prevention and treatment.

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The use, production and purification of the egg yolk IgY is known as IgY technology [6]. IgY is the only immunoglobulin isotype present in the egg yolk, the main serum immunoglobulin in birds and it is considered to be

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ancestral of mammalian IgG and IgE [7]. IgY technology has several advantages over conventional mammalian antibody production: chicken housing is inexpensive, egg collection is non-invasive and IgY isolation is relatively fast and simple [8]. Considering that large scale production of mammalian antibodies is expensive and uneasy, egg yolk antibodies represent a low-cost and feasible alternative [9].
The aim of this work was to obtain specific IgY antibodies against BRSV capable of neutralizing infection *in vitro*. These antibodies could be potentially used in passive immunization studies.

2. Materials and Methods

2.1. Virus and cells

The A51908 BRSV strain was propagated on MDBK cells, grown in a mixture of equal parts of E-MEM (Eagle´s minimal essential medium) and D-MEM (Dulbecco´s minimal essential medium) (GIBCO BRL) containing 5% of foetal bovine serum (FBS) (Internegocios) free of antibodies against BRSV. Flasks containing MDBK monolayers with 90% confluence were infected with BRSV at a MOI of 0.1. After viral adsorption, cells were incubated at 37ºC in a 5% CO_2 atmosphere. Five days after infection (DPI), when the cytopathic effect (CPE) was greater than 80%, the virus was harvested by a freeze-thaw cycle, followed by a centrifugation step at 3250 x g. The supernatant was stored at -70ºC until use.

2.2. Viral concentration

BRSV concentration was performed using a precipitation method with polyethylene glycol (PEG) 6000 according to Gias et al. [10]. Briefly, the virus obtained from MDBK culture was clarified by centrifugation at 3250 × g for 20 minutes at 4°C. The clarified viral supernatant was then adjusted to 100 mM $MgSO_4$ and 50 mM Tris-HCl (pH 7.5). Fifty per cent (wt/v) PEG 6000 in NT buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5) was added to the clarified culture fluid to a final concentration of 10% (v/v). The virus particles were then precipitated for 90 minutes at 4°C with moderate stirring, followed by centrifugation at 3250 × *g* for 20 minutes at 4°C. After removing the supernatant, the pellet was centrifuged as above and the residual fluids were removed. The pellet was then re-suspended in a volume of cold NT buffer equal to 2% of the initial volume of virus and frozen at -70ºC until use.

2.3. Viral titration

The infective titer of viral productions was determined by CPE using the Reed and Muench method [11].
Titration was performed in MDBK cells grown in 96-wells plates (NUNC). Cell monolayers were inoculated in quadruplicate with 100 µl of seven tenfold dilutions of BRSV. Plates were incubated for 5 days at 37ºC with 5% CO_2 atmosphere. Infective titers were expressed as Tissue Infective Culture Dose 50/ml (TICD50/ml), defined as the dilution of viral suspension needed to infect 50% of cells.

2.4. Serum neutralization assay

Serum neutralization assay was carried out as described previously by Samal et al. [12]. Inactivated serum samples were fourfold diluted from 1:4 to 1:256. The serum-dilutions were supplied with 100 TCID50 of a A 51908 BRSV strain and incubated for 1 hour at 37ºC in a 5% CO_2 atmosphere. This mixture was inoculated in duplicate onto MDBK cell monolayers (250000 cells/ml) in 96-well plates. Plates were incubated as above and CPE was observed 5 DPI.
Samples were considered positive when no CPE was observed. Antibody titers were expressed as the reciprocal of the maximum dilution in which no CPE was observed. Samples with titers higher than 4 were considered positive.

2.5. Production of IgY against BRSV

2.5.1 Hens immunization

Three light brown laying hens (A, B and C), seronegative for BRSV, were immunized intramuscularly in the breast muscle with 0.5 ml of immunogen. In the first dose, birds received 0.2 ml of antigen subcutaneously in the neck. Vaccines were prepared with Freund´s complete adjuvant, for the first dose, and with Freund´s incomplete adjuvant for the following doses, containing the antigen in a proportion adjuvant:antigen of 50:50.
Two experiences were performed.

- Experience I: hens A and B received 6 doses of an immunogen containing 10^5 TCID50/ml BRSV at days 0, 18, 32, 56, 85 and 106.
- Experience II: hen C received 2 doses of an immunogen containing 10^7 TCID50/ml BRSV at days 0 and 42.

2.5.2. Blood sampling and eggs collection

One week after each immunization serum samples were obtained from immunized hens. In the first experience, hens were bled at days 0, 7, 24, 39, 64, 96 and 119, and in the second experience blood samples were obtained at days 0, 13, 50 and 70. Serum samples were stored at -20ºC until use.

In the first experience, eggs were collected at days 0, 12, 30, 44, 70, 97, 103, 118, 123 and 128; and in the second experience at days 0, 7, 13, 51, 57 and 70.

Hens´ management, inoculation and sample collection were conducted by trained personnel under the supervision of a veterinarian and in accordance with protocols approved by the INTA's ethical committee of animal welfare.

2.6. Purification of Egg Yolk IgY

IgY from egg yolk was purified according to Akita and Nakai [8]. Briefly, the yolks were diluted with 5 volumes of distilled water and then frozen at -20°C. Thereafter, diluted yolks were thawed at 4°C over night (ON) and centrifuged at 8000 x g for 12 minutes at 4°C. The pellet was discarded and 0.24 g/ml of $(NH_4)_2SO_4$ were added to the supernatant, incubated for 30 minutes at room temperature with slightly stirring and then centrifuged at 10000 x g for 12 minutes at 4°C. The pellet was re-suspended in $(NH_4)_2SO_4$ 2M, for 30 minutes at room temperature and centrifuged as above. Finally, the supernatant was discarded, the pellet was re-suspended in phosphate-buffered saline (PBS) and then the solution was dialyzed ON against PBS at 4°C.

2.7. SDS-PAGE and western blot (WB)

Purified IgY were electrophoresed on SDS-PAGE (12%) and stained with Coomassie blue.

To test the identity of the purified IgY anti-BRSV, a direct WB was performed. Yolk extracts and purified IgY were subjected to SDS-PAGE (12%), blotted onto an Immobilon P membrane (Millipore) and blocked ON with PBS Tween 0.1% (PBS-T) and skim milk 3%. All subsequent steps were carried out using this buffer. The membrane was then incubated 1 hour at 37°C with the purified IgY solution, washed three times and finally incubated 1 hour at 37°C with an antibody horseradish peroxidase-labelled goat anti-chicken IgY (Sigma®). After washing three times, the WB was developed by DAB/H_2O_2 system (Sigma®). As negative control a purified bovine IgG was used (Bovine IgG Bethyl).

2.8. Dot blot

The antigen preparation was performed according to a protocol adapted from Lerch et al. [13]. Briefly, a monolayer of MDBK cells contained in a T75 flask was infected with a viral stock of BRSV (MOI=0.3). Five (DPI), the medium was removed and the monolayer was rinsed with 5 ml of PBS 1X. Cells were harvested into lysing buffer (1% [v/v] Nonidet P-40, 0.4% [wt/v] deoxycholic acid, 66 mM EDTA, and 10 mM Tris-Cl pH 7.4). The nuclei were removed by centrifugation at 2000 x g for 10 minutes at 4ºC and the cytoplasmatic extract was made 0.2% (wt/v) with SDS.

Negative control (mock infected MDBK cells) was also prepared by the same procedure. Both antigen and mock were stored at -70ºC until use.

PVDF membrane (polyvinylidene fluoride, Millipore Corporation) strips were cut into strips of 1 cm each one. On each segment 1μl of a viral suspension of 10^5 FFU/ml and mock were immobilized at different dilutions in TBS-T 0.1%: 1: 64, 1:128 and 1:256 (for serum IgY) and 1:32, 1:64, 1:128 (for egg yolk IgY). A non-labelled antibody anti-IgY (Sigma®), which captures specifically IgY, was used as positive control of the technique, diluted in a proportion 1:100 in TBS-T 0.1%. The membranes were dried at room temperature for 20 minutes and then were blocked for 1 hour at 37°C with blocking buffer (TBS-Tween 20 0.1% - skim milk 10% wt/v). Membranes were adsorbed with mock infected MDBK cells, for 1 hour at 37°C with slightly stirring. Then, the membranes were incubated for 1 hour at 37°C with different dilutions of IgY (1:5120, 1:10240, 1:20480 and 1:40960). Membranes were washed 4 times with TBS-T 0.1% for 10 minutes at 37°C and were incubated with an antibody horseradish peroxidase-labelled goat anti-chicken IgY (Sigma®) at a 1:4000 dilution in blocking buffer for 1 hour at 37°C. Finally, the membranes were washed 4 times with TBS-T 0.1% and once with TBS 1X. The reaction was developed with DAB/H_2O_2 system (Sigma®) and stopped with distilled water.

3. Results and Discussion

Several BRSV vaccines have been commercialized for many years, however, it has not been possible to eradicate the disease caused by this virus. It has been reported that animals that had been previously infected or vaccinated could

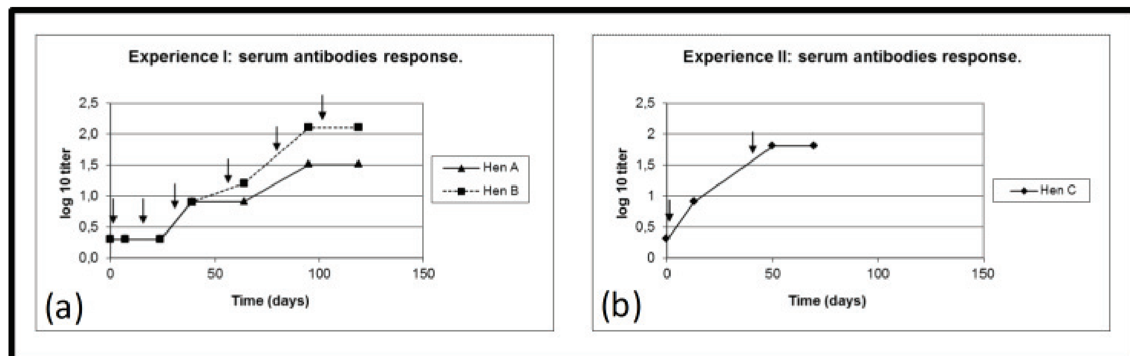
be reinfected with BRSV even from the same strain. Additionally, no vaccines are yet available against HRSV. This poses the need to develop alternative and complementary strategies to reduce viral infection and for the treatment of the disease. Passive immunization appears as an interesting strategy to prevent respiratory viral infections both in human and cattle. In this sense, IgY technology presents several advantages over conventional antibodies production systems.

In this work, hens were immunized with BRSV in order to obtain specific antibodies that could neutralize viral infection.

The kinetic of serum antibodies from inoculated animals was analyzed by a serum neutralization assay. In the first experience, birds began to respond after the third immunization and antibody titers continued to increase to a maximum of 1.5 for hen A and 2.1 for hen B, after the fifth immunization (Figure 1 a).

In order to reduce the number of immunizations and to improve the immune response, a second experience was conducted, in which hens were immunized with concentrated BRSV. In this experience, it was possible to detect serum antibodies against BRSV after the first immunization and high titers were detected after the second immunization (Figure 1 b).

Figure 1: Kinetic of serum antibodies measured by serum neutralization assay.



Neutralization titres of IgY from experience I (a) and II (b) are expressed as the reciprocal of the highest dilution showing no CPE. The arrows indicate the inoculation times.

It is important to note that in the second experience, only two doses were enough to induce specific neutralizing antibodies, reducing suffer and stress of birds caused by repeated inoculations, according to the 3R´s principle (Reduce, Refine and Replace) [14].

The presence of IgY in the egg yolks after purification was confirmed by SDS-PAGE followed by western blot analysis. There was a significant reduction in the number of bands in the purified samples. The band corresponding to serum albumin protein observed in samples prior to purification process was eliminated. It is important to eliminate this protein given that, along with transferrin and lysozyme present in the egg white, is responsible for the allergenic egg properties. The final concentration of IgY was 21.7 mg/ml.The two major bands in purified IgY representing the heavy (70 kDa) and light (25 kDa) chains of IgY were confirmed by a direct western bot assay (Figure 2 a and b).

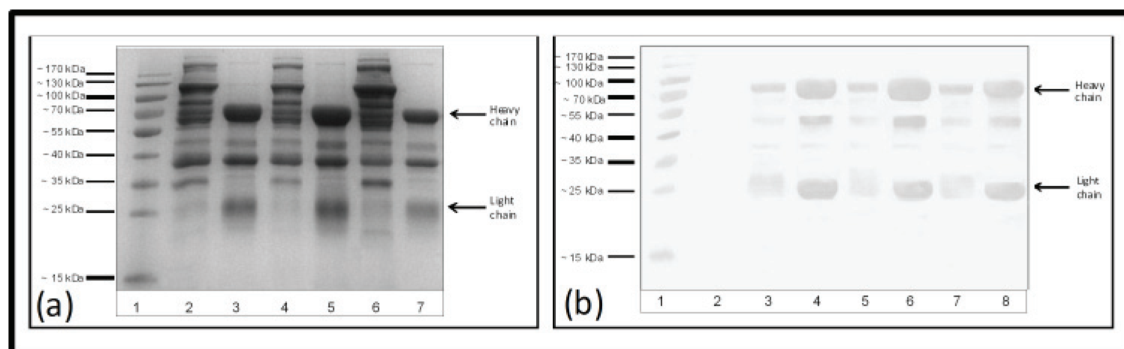


Figure 2: Detection of IgY in the egg yolk.

(a) SDS-PAGE (12%) of egg yolk IgY. (1) MWM, (2) unpurified egg yolk from hen A, (3) purified IgY hen A, (4) unpurified egg yolk from hen B, (5) purified IgY from hen B, (6) unpurified egg yolk from hen C, (7) purified IgY from hen C.

(b) Western blot. (1) MWM, (2) bovine IgG, (3) unpurified egg yolk from hen A, (4) purified IgY from hen A, (5) unpurified egg yolk from hen B, (6) purified IgY from hen B, (7) unpurified egg yolk from hen C, (8) purified IgY from hen C.

The specificity of the IgY obtained against BRSV in hen B, which presented the highest serum neutralizing antibody levels, was evaluated by a dot blot assay. IgY antibodies were able to specifically recognize the virus at a dilution up to 1:20480 (Figure 3).

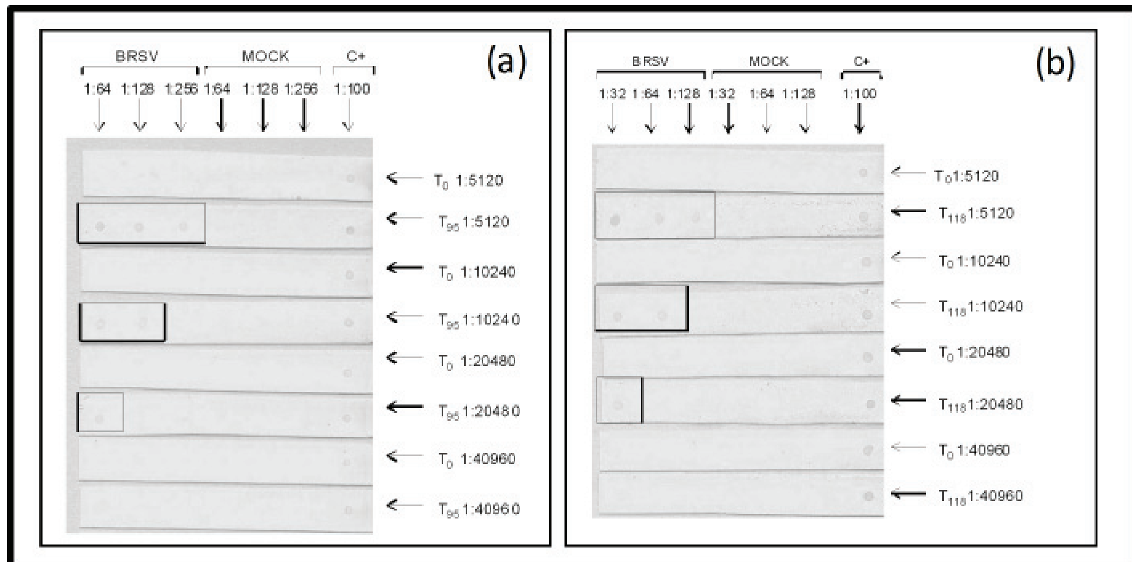


Figure 3: Detection of specific IgY in serum and egg yolk.
Serum IgY (a) and egg yolk IgY (b) from hen B against BRSV were measured by dot blot assay as described in Materials and Methods. Specific IgY were evaluated at the beginning of the experience (To) and 95 or 118 days after the first immunization in serum and egg extract, respectively.

In order to evaluate the ability of egg yolk antibodies to neutralize infection *in vitro,* a neutralization test was performed. In the first experience (Figure 4 a), it was possible to detect BRSV neutralizing antibodies after the third inoculation, reaching the highest titer, for both chickens, after the fifth dose; while in the second experience, the hen C developed neutralizing antibodies after the second inoculation (Figure 4 b). In this case, the immune response could be improved by increasing the mass of the antigen used for inoculations.

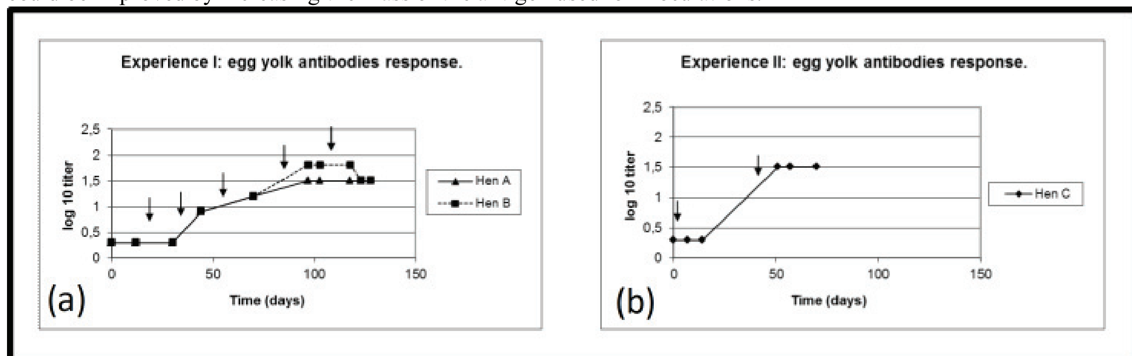


Figure 4: Evaluation of the neutralizing activity of the purify IgY.
Kinetic of egg yolk antibodies from experience I (a) and II (b). Titers are expressed as the reciprocal of the highest dilution where no CPE was detectable. Arrows indicate the inoculation times.

In this work it was possible to obtain IgY antibodies against BRSV that could specifically recognize the virus. Moreover, these antibodies were able to neutralize infection *in vitro*. IgY technology represents an attractive tool that could be potentially used in prophylaxis and/or treatment of respiratory disease caused by BRSV infection.

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