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Passive protection effect of chicken egg yolk immunoglobulins on enterovirus 71 infected mice

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ABSTRACT

The objective of this study is to evaluate the passive protective efficiency of immunoglobulin in yolk (IgY) specific against human enterovirus type 71 (EV71). The antibody was raised by intramuscular immunization to 10 White Leghorn hens, with inactivated human EV71 serving as the antigen. The titer and specificity of the antibody were analyzed from purified IgY in the egg yolks of immunized hens. Results indicate that the titer of IgY specific against EV71 increased from the third week after the first immunization. The content of total IgY was 190 ± 26 mg/yolk, with an average concentration of specific IgY of 6.34 ± 3.38 mg/yolk in the eggs from 3 to 18 wk after immunization. The results of the neutralization effect of specific IgY in EV71-challenged mice demonstrate that the EV71-specific IgY, either by intraperitoneal injection or oral administration, was able to significantly reduce the morbidity and mortality in EV71 infected mice pups.

1. Introduction

Researchers have extensively studied passive immunization in both humans and animals using specific antibodies to protect against pathogens. An increase in antibiotic-resistant bacteria and the desire to treat pathogens that do not respond to antibiotics, such as viral pathogens, has prompted many researches to use antibodies as alternative to antibiotics [1]. IgY (immunoglobulin in yolk) antibodies are the predominant serum immunoglobulin in birds, reptiles, and amphibians, and are transferred from serum to egg yolk in the females to confer passive immunity to their embryos and neonates [2]. The potential of orally administered IgY for the prevention and treatment of many pathogens has been studied for many years, for *Escherichia coli* [3], *Helicobacter pylori* [4], *Salmonella* [5], *Rotavirus* [6], *Staphylococcus* [7], *Streptococcus mutans* [8], *Yersinia* [9], *Coronavirus* [10], and the *Porcine epidemic diarrhea* virus [11]. This paper evaluates the efficiency of IgY against enterovirus 71 (EV71).

Enterovirus 71 belongs to the human enterovirus A family of *Picornaviridae*. These virions consist of a non-enveloped capsid surrounding a core of single-stranded, positive-polarity RNA approximately 7.5 kb in size [12]. Since the initial description of EV71 in 1974 [13], outbreaks of this virus have been identified periodically in countries throughout the world, including the USA, Australia, Sweden, Japan, Bulgaria, Hungary, Hong Kong, and Malaysia [14]. In 1998, an outbreak of EV71 infections occurred in Taiwan, in which 405 children were hospitalized and 78 died [15]. EV71 infections are generally mild, like hand-foot-and-mouth disease (HFMD) and herpangina, but occasionally lead to severe diseases such as aseptic meningitis, poliomyelitis-like paralysis, and even fatal encephalitis in neonates [16–18]. In recent years, some efforts have been made to control EV71 infections. The most promising antiviral agents for EV71 are WIN-group compounds, which have undergone clinical trials [19,20]. In addition, a research group from Taiwan has developed two candidate EV71 vaccines, including a formalin-inactivated whole virus vaccine and a VP1 expressing DNA vaccine [21]. The efficacy of both vaccine constructs is currently being tested in animal models.

Several approaches have been attempted in the treatment of viral diseases. Researchers tried to block the replication of virus through binding the capsid of virus with some chemicals, such as pyridazinamines [22], phenoxyl imidazoles [23], or pleconaril [24,25]. However, the treatment of EV71 with pleconaril was not significant, the binding of pleconaril with capsid was limited under higher viral concentration *in vitro* [26]. A specific antibody which could bind with the virus and hence reduce the contact of virus with host cells is an alternative choice. Human's immunoglobulin has been applied in the treatment of EV71 infections in babies whose immune system are not well-developed [27,28]. There is no routine therapy for the treatment of EV71 infection with human's

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immunoglobulin so far. However, some animal model of EV71 challenged mouse has been developed, and provided some information about the protective effects of the neutralizing antibody on EV71 infection [29]. Compared to traditional antibody production, chicken as bio-factory can produce higher yield of IgY antibodies than mammals' IgG. A chicken can lay 280 eggs in a year and an egg yolk contains 100–200 mg of IgY antibodies.

This study was subjected to produce IgY against enterovirus 71 (anti-EV71 IgY) and evaluated the inhibition effects of specific IgY on EV71, including *in vitro* virus neutralization test and *in vivo* ICR mice model. This study also provided an animal model for the application of IgY in the cure or prevention of EV71.

2. Materials and methods

2.1. Virus strains and cells

The EV71 strains 4643 and MP4. Strain 4643 was originally derived from a patient with EV71 encephalitis [30], while strain MP4 was a mouse-adapted enterovirus 71 with increased virulence in mice, and it was generated after four serial passages of the 4643 strain [31]. EV71 stock virus of strains 4643 and MP4 were grown in RD (rhabdomyosarcoma) cells, which were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, U.S.A.) containing 10% fetal bovine serum (FBS, Invitrogen, U.S.A.), 2 mM of L-glutamine (Invitrogen, U.S.A.), 100 IU/ml of penicillin (Invitrogen, U.S.A.), and 100 μg/ml of streptomycin (Invitrogen, U.S.A.).

2.2. Immunization of chickens

All animals received humane care as outlined in the guide for the care and use of experimental animals and viral challenge (Institutional Animal Care and Use Committee; IACUC Approval No. 940020). Four 6-month-old White Leghorn laying hens, obtained from Livestock Research Institute, Council of Agriculture, Executive Yuan, Taiwan, were utilized for the production of anti-EV71 IgY. A formalin-inactivated EV71 of strain 4643 was utilized as the antigen. One milliliter of EV71 antigen (400 μg/ml, 5.4×10^6 pfu) was emulsified with an equal volume of Freund complete adjuvant and immunized intramuscularly to chickens at 4 sites in the breast muscle. Four booster injections with Freund's incomplete adjuvant were given at weeks 2, 4, 6, and 13 after the first immunization. The eggs were collected daily from the first day to 3 weeks after the last immunization, and stored at 4 °C. The egg yolk was separated, pooled, and kept at −20 °C prior to IgY purification, and all egg yolks from each chicken for each week were pooled into one analysis sample.

2.3. Isolation and purification of IgY

The isolation of IgY was carried out as described by Akita and Nakai [32,33], with some modifications. The egg yolk was first mixed with one of nine volumes of cold distilled water (acidified with 0.1 N HCl to pH 5.0) and stored overnight at 4 °C. The mixture was then centrifuged at $3125 \times g$ at 4 °C for 40 min to obtain the water soluble fraction (WSF). The WSF was collected and filtered to remove solids. The resulting IgY-containing WSF was further purified by ultra filtration using Amicon Ultra-15 filter (PL-100, Millipore), condensing the sample to 1/30 to 1/40 of its original volume. These WSF concentrates were then subjected to the virus neutralization test and mice challenge experiments.

2.4. ELISA (enzyme-linked immunosorbent assay)

The antibody activity of anti-EV71 IgY was determined using the ELISA method described by Lee et al. [34], with some modifications. Briefly, microtiter plates were coated with 100 μl of inactivated EV71 4643 antigen (10 μg/well), while control wells were coated with rabbit anti-chicken IgY antibody (10 μg/ml, Sigma C2288). The plate was then incubated overnight at 4 °C. After washing with PBS–Tween 20 buffer, 2% BSA blocking was conducted overnight at 4 °C. The wells were then washed with PBS–Tween 20 buffer. Next, 100-fold diluted WSF was added to the sample wells (100 μl/well) for testing. WSF from the same chicken before immunization was used as a control. To generate the standard curve, wells were filled with 100 μl serial-diluted pure chicken IgY at a concentration from 0.015 μg/ml to 1 μg/ml (Promega, G116A) and incubated at 4 °C for overnight. After washing with PBS–Tween 20 buffer, 100 μl of alkaline phosphate-conjugated goat anti-chicken IgY (Promega, G115A) was added to the wells and incubated at 37 °C for 2 h. After washing with PBS–Tween 20 buffer, 100 μl of disodium p-nitrophenyl phosphate was added to each well as a substrate (Sigma, N9389) and allowed to react at 37 °C for 10 min. The absorbance was then measured at 405 nm using a microplate reader (Multiskan MS: Thermo Labsystems). The resulting absorbance of standard curves provided a relative measurement of anti-EV71 IgY concentration. For the total IgY determination, each well on the microtiter plate was first coated with 100 μl of rabbit anti-chicken IgY antibody (10 μg/ml, Sigma C2288), to which 100 μl of 10,000-fold diluted WSF was then added. The following experiments were performed following the same protocol described above.

2.5. Western blot analysis

The EV71 was run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrically onto a nitrocellulose membrane. The membrane was divided into 3 parts and soaked in blocking buffer (5% nonfat dry milk in TBST) for 30 min at room temperature, then incubated with anti-EV71 or non-specific IgY for 1 h. After washing 4 times with TBS-T, the membranes were incubated with peroxidase-conjugate goat anti-chicken IgY (1000-fold dilution in blocking buffer for each) at room temperature for 1 h. Immunoreactivity was detected by incubating the membranes with 0.02% (W/V) 4-methoxy-1-naphthol in TBS and 0.02% (V/V) H_2O_2.

2.6. $TCID_{50}$ and EV71 neutralization tests

The $TCID_{50}$ of MP4 virus and the neutralization titer of anti-EV71 IgY were determined using the method described by Yu et al. [35]. In the $TCID_{50}$ tests, each 96-well 8×10^3 RD cell was added to a microplate well, followed by the 10-fold serial diluted virus. The 50% tissue culture infective dose was determined after incubation under 5% CO_2 at 37 °C for 7 days. In the neutralization tests, each 50 μl sample of serial diluted anti-EV71 IgY concentrate solution was mixed with 50 μl of 100 $TCID_{50}$ EV71 in a microplate well. Two hours later, the RD cell suspensions (8×10^3 cell/well) were added and further incubated under 37 °C and 5% CO_2 for 7 days. Neutralization antibody titers were then identified as the highest dilution of purified IgY solution that completely inhibited virus growth.

2.7. EV71 infection and IgY protection tests

The challenge test of EV71 of the mouse model in this study was modified from Wang et al. [30]. Trials 1–4 were designed to test the effect of IgY with different neutralization titers on mortality in suckling mice. Each one-day-old ICR strain mouse was intraperitoneally (IP) inoculated with 1×10^5 pfu of MP4 virus, which is a mouse-adapted 4643 strain of EV71. Thereafter, IgY with different neutralization titers (64, 128, 256, and 512) was injected IP into the challenged mice 1–3 days after inoculation (dpi). The dosage of IgY

Table 1
Purity of IgY after different procedure of purification.

Purification step	Total protein (mg/egg)	IgY (mg/egg)	Purity (%)
Water soluble solution (WSF)	595 ± 37[a]	190 ± 26[a]	32 ± 6
Ultrafiltration	231 ± 25[a]	151 ± 21[a]	65 ± 5

[a] The value was derived from the concentration of the water soluble fraction (WSF), based on an average WSF of 150 ml per egg from all chicken eggs between 3 and 18 weeks, was mean as average ± SD.

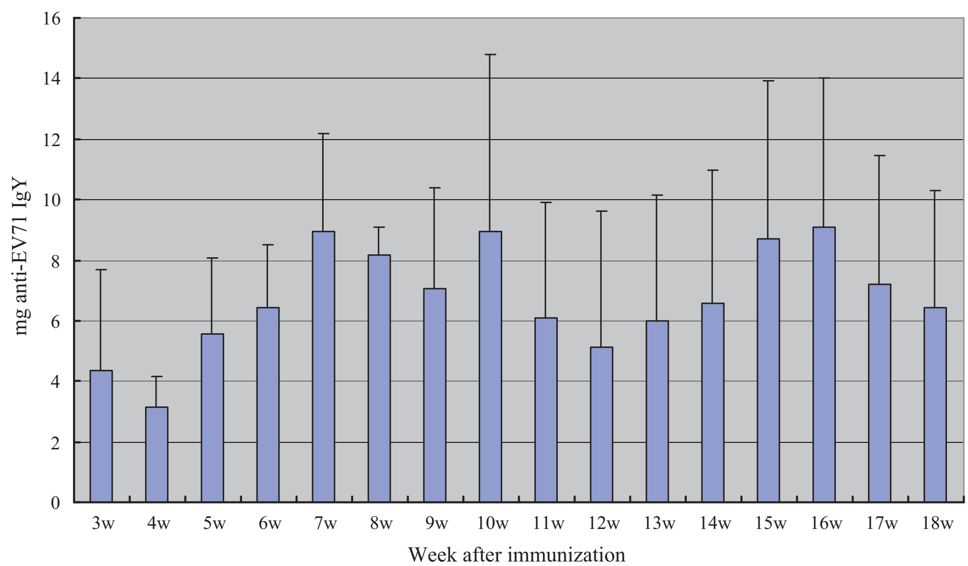

Fig. 1. The content of EV71 specific IgY per egg yolk during the immunization period. Values are shown as average data from eggs in the same week for all immunized chickens. Vertical bars indicate the standard deviation.

was 100 μl per mouse. In trials 5 and 6, the IgY treatment dates were 2–4 dpi and 4–6 dpi, respectively. Trials 7–8 were used to evaluate the protection effect of anti-EV71 IgY by oral treatment. Specifically, trials 7 and 8 used a 24-gauge feeding tube to orally inoculate mice of 4–5 d (2.5–3.0 g) and 5–6 d old (3.0–3.5 g), respectively. The mice were given 100 μl of MP4 virus (3×10^6 pfu/mouse) after 12-h of fasting. For each pup, 100 μl of anti-EV71 IgY with a titer of 512 was orally administered either 1 h before or 1 h after viral challenge. All mice were checked daily for their body weight and the syndromes of EV71 infection, including limb paralysis and abnormal hair coat until 2 weeks after viral infection. Morbidity and mortality data was collected for two weeks after the viral challenge.

3. Results

3.1. Production of IgY against EV71

The concentration of total IgY and specific anti-EV71 IgY was analyzed by ELISA. The content of IgY in the WSF of egg yolk was investigated for a period of 18 weeks, from the first week of immunization until 6 weeks after the last booster injection. The average content of total IgY in each yolk was 190 ± 26 mg (Table 1), which was 32% of the egg yolk's total protein. After condensation by ultrafiltration, the total protein per egg was 231 ± 25 mg on average, and the purity of total IgY increased to 65%.

The content of specific anti-EV71 IgY increased gradually from the third week after the first immunization, as Fig. 1 indicates. A variation in immune response among individual hens was noticed from the large standard deviation. The average anti-EV71 IgY concentration reached 6.34 ± 3.38 mg per yolk between the third to eighteenth weeks, equivalent to 3.33% of the total IgY.

3.2. The neutralization titer determination of specific IgY

The neutralization titer of specific anti-EV71 IgY was determined by the cytopathic effect (CPE) of EV71 on RD cells (Fig. 2). The resulting neutralization titers of specific IgY obtained from eggs of all immunized chickens ranged from 0 to 2048 (data not shown). Comparing the neutralization titers to the contents of specific IgY in the respective yolks showed no significant cor-

Table 2
Neutralization titers of EV71 specific IgY from different chickens.

Chickens	Concentration of specific IgY against EV71[a] (μg/ml)	Neutralization titer[b]
YA1 (8W)	9.6	0
YA2 (17W)	33.4	16
YA3 (15W)	37.8	0
YA4 (8W)	45.5	0
YB1 (6W)	40.1	0
YB2 (8W)	1.7	64
YB3 (9W)	50.2	512
YC1 (15W)	28.9	64
YC2 (15W)	19.9	256
YC3 (8W)	16.8	0
YC4 (8W)	20.7	16
YE1 (16W)	49.1	2048
YE2 (11W)	46.5	256
YE3 (15W)	123	16
YE4 (7W)	66.5	128

[a] The concentration of WSF specific IgY against EV71 was determined using the ELISA method.
[b] The IgY-containing WSF was further purified by ultra filtration using Amicon Ultra-15 filter (PL-100, Millipore), condensing the sample to 1/40 of its original volume. These WSF concentrates were then subjected to the virus neutralization test.

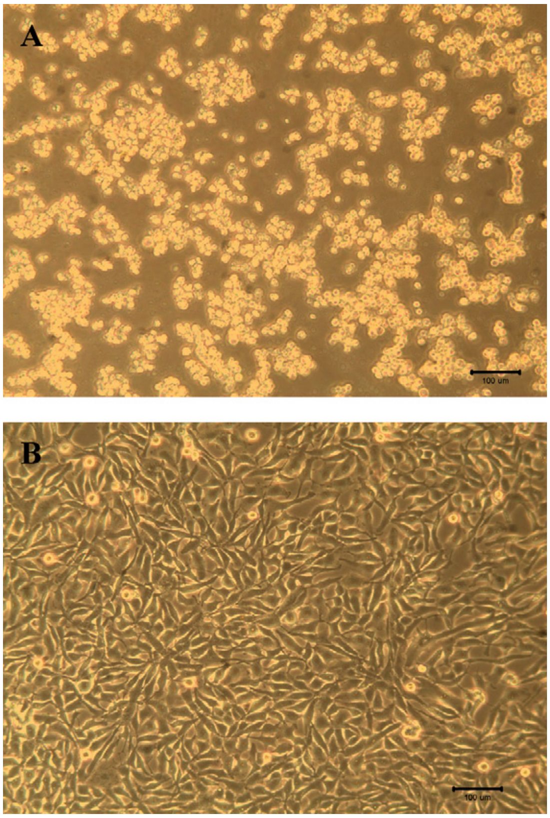

Fig. 2. (A) The transfection of EV71 on RD cells showing the cytopathic effect (CPE). (B) RD cells showed no CPE if anti-EV71 IgY was mixed with EV71 for 2 h, indicated virus neutralization. Scale site shows 100 μm at 100×.

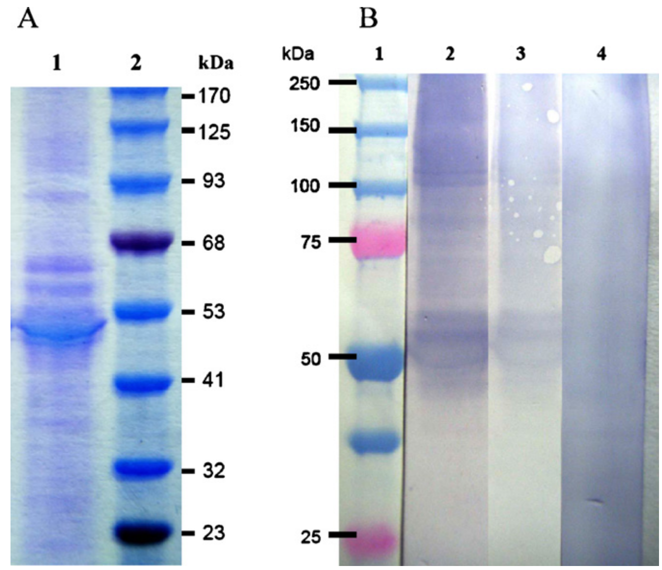

Fig. 3. Western blot analysis of anti-EV71 IgY. (A) SDS-PAGE profiles of EV71 (lane 1: EV71 virus, lane 2: molecular maker) and (B) Western blotting analysis of the immunoreactivity of anti-EV71 IgY. The EV71 was run SDS-PAGE and transferred onto nitrocellulose filter and probe with anti-EV71 IgY and non-immunized IgY (lane 1: molecular maker; lane 2: anti-EV71 IgY 1:1000 dilution: lane 3: anti-EV71 IgY 1:3000 dilution; lane 4: non-immunized IgY).

relation between these two sets of data ($r = -0.396$, data not shown).

Western blotting analysis of the immunoreactivity of anti-EV71 IgY. The EV71 was run SDS-PAGE and transferred onto nitrocellulose filter and probe with EV71 immunized and non-immunized IgY. The EV71 was detected of ∼52 kDa molecular mass (Fig. 3A) and anti-EV71 IgY was specifically bound to the proteins witch molecular weight corresponding of EV71 virus (Fig. 3B).

3.3. Neutralization effect of specific IgY in EV71 challenged mice

In the first part of the animal tests, ICR mice showed both limb paralysis and abnormal hair growth after EV71 challenge in the IP model (Fig. 4). In trials 1–6 was conducted to investigate the effect of IP injected specific IgY on curing IP EV71-challenged mice (Table 3). When one-day-old ICR mice were challenged with EV71 of strain MP4 with a dose of 10^5 pfu per mouse, the average morbidity was 96.5 ± 6.1% in the control group. The resulting mortality was 88.0 ± 8.4% based on the challenged mice, or 91.2 ± 7.6% when based on illness in mice injected with only non-immunized IgY (Table 3). We IP administrated the purified specific IgY derived from condensation of the immunized yolk WSF to EV71-challenged mice. This administrated was applied continuously for 3 days, from the day after viral infection (1 dpi) to the third day post infection (3 dpi). The therapeutic effects of IgY varied according to the neutralization titers of the specific IgY administered. The infection rate was 100% when the titer of specific IgY was 64, which is same infection rate as in the control group (Table 3). The treatment of IgY with a titer of 64 did not significantly reduce mortality in trial 1. However, when the IgY titers were 128 or higher, the morbidities reduced to 20%, 7%, and 0%, respectively for IgY treated groups with titers of 128, 256 or 512. Only the group of titer 128 (trial 2) had some deaths after viral infection and IgY injections, with a mortality rate of 10%. Without the treatment of specific IgY, the challenged mice showed high morbidities and mortality. In trials 5 and 6, if the treatment

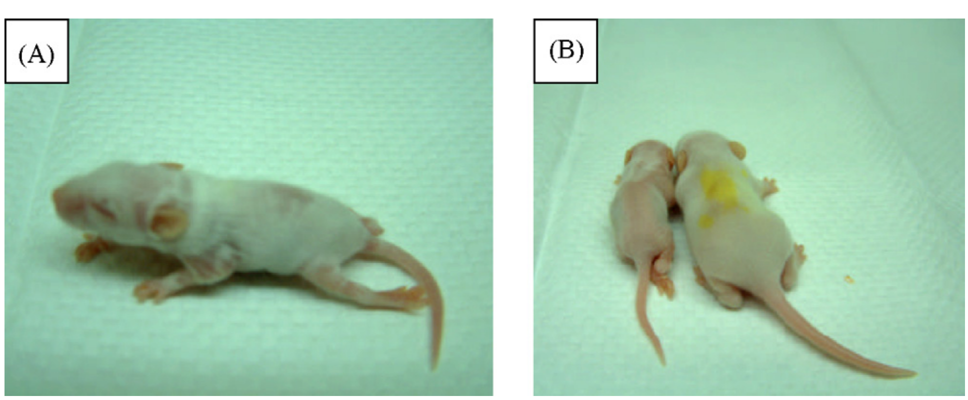

Fig. 4. ICR mice showed both limb paralysis and abnormal hair growth after EV71 challenge in the IP model. (A) Mouse of 8 dpi. (B) Body weight difference between an EV71 infected mouse (left) and anti-EV71 IgY treated mice (right) on 8 dpi at the same cage.

Table 3
Effects of IP challenge on EV71[a] and IgY[b] treatment.

Trial no.	Mice no.	EV71 specific IgY titer	IgY IP date (dpi)	Morbidity (%)	Mortality (%)	Mortality of ill mice (%)
1	14	64	1–3	100(14/14)	92(13/14)	92(13/14)
Control[c]	13	64	1–3	100(13/13)	77(10/13)	77(10/13)
2	10	128	1–3	20(2/10)[#]	10(1/10)[#]	50(1/2)
Control[c]	12	128	1–3	100(12/12)	100(12/12)	100(12/12)
3	30	256	1–3	7(2/30)[#]	0(0/30)[#]	0(0/2)
Control[c]	26	256	1–3	100(26/26)	92(24/26)	92(24/26)
4	21	512	1–3	0(0/21)[#]	0(0/21)[#]	0(0/21)
Control[c]	23	512	1–3	100(23/23)	91(21/23)	91(21/23)
5	14	512	2–4	43(6/14)[#]	21(3/14)[#]	50(3/6)
Control[c]	16	512	2–4	94(15/16)	88(14/16)	93(14/15)
6	19	512	3–5	89(17/19)	63(12/19)	71(12/17)
Control[c]	20	512	3–5	85(17/20)	80(16/20)	94(16/17)
Trial (Ave ± Std)				43.1 ± 42.5[#]	31 ± 37.9[#]	34.2 ± 32.4
Control (Ave ± Std)				96.5 ± 6.1	88.0 ± 8.4	91.2 ± 7.6

[a] IP infection dose: 10^5 pfu/mouse, one-day-old mouse.
[b] IP injection dose: 100 μl/mouse/day (IgY titer between 64 and 512).
[c] Non-immunized IgY.
[#] Differences in proportions morbidity and mortality were tested with the use of the X^2 statistic ($P < 0.05$).

Table 4
Effects of oral challenge on EV71[a] and IgY[b] treatment.

Trial no.	Mice no.	Age and BW	Oral IgY ad. time	Morbidity (%)	Mortality (%)	Mortality of ill mice (%)
7-1	25	4–5 D	1 h before	24(6/25)[#]	20(5/25)[#]	91(5/6)
Control	26	2.5–	1 h before	69(18/26)	62(16/26)	89(16/18)
7-2	16	3.0 g	1 h after	38(6/16)[#]	31(5/16)[#]	83(5/6)
Control	22		1 h after	91(20/22)	77(17/22)	80(16/20)
Trial (Ave ± Std)				31.0 ± 9.9[#]	25.5 ± 7.8[#]	87.0 ± 5.7
Control (Ave ± Std)				80.0 ± 15.6	69.5 ± 10.6	84.5 ± 6.4
8-1	19	5–6 D	1 h before	10(2/19)	5(1/19)	50(1/2)
Control	19	3.0–	1 h before	32(6/19)	32(6/19)	100(6/6)
8-2	19	3.5 g	1 h after	16(3/19)	11(2/19)	67(2/3)
Control	19		1 h after	26(5/19)	21(4/19)	80(4/5)
Trial (Ave ± Std)				13.0 ± 4.2	8.0 ± 4.2	58.5 ± 12.0
Control (Ave ± Std)				29.0 ± 4.2	26.5 ± 7.8	90.0 ± 14.1

[a] Oral inoculation dose: 3 × 106 pfu/mouse.
[b] Orally administrated IgY dose: neutralization titer 512, 100 μl/mouse.
[#] Differences in proportions of morbidity and mortality were tested with the use of the X^2 statistic ($P < 0.05$).

time of specific IgY began at 2 or 3 dpi, the morbidities increased to 43% and 89%, respectively. This reduction in cure effects increased mortalities by 21% and 63%, respectively. The mortalities of mice showing illness were 50% and 71% in trials 5 and 6, respectively, indicating a decrease in cure effects of specific IgY following the delay of IgY administration for one or 2 days.

The second part of the animal tests involved oral administration of specific IgY to EV71 orally challenged mice. In trial 7, a dosage of 3×10^6 pfu MP4 strain EV71 was orally fed to pups 4 or 5 days old with a body weight of 2.5–3.0 g. The results indicated that if 100 μl of specific IgY was orally fed 1 h before the viral challenge, the morbidity and mortality of challenged mice was 24% and 20%, respectively. Both morbidity and mortality were significantly reduced compared to those of the control group, at 69% and 62%, respectively (Table 4). When the specific IgY was given to the pups 1 h after the viral challenge, the infection rate was 38% with a final mortality rate of 31%. The morbidity and mortality rates of the specific IgY treatment groups were lower than those of the control group. Regardless of whether the IgY was fed 1 h before or after the viral challenge, virus neutralization by specific IgY was effective. Comparing the mortality of mice exhibiting infection symptoms, the IgY treated groups showed a similar rate to the control groups, which all exceeded 80%. The IgY application in trial 8 was similar to that in trial 7, changing only the age of challenged pups from 5- to 6-days old, with their body weight ranging from 3.0 to 3.5 g. Under this situation, the average infection rate decreased to 13% in the IgY treated group and 29% in the control group. Also, the mortality in the IgY treated group among ill pups was lower than that in the control groups. Although the average mortality of IgY treated groups was as low as 8%, the control groups also exhibited a lower mortality (26.5%) than the control groups in trial 7.

4. Discussion

In recent years, pathogen-specific IgY has also been demonstrated to be effective in the passive protection of human's diseases, such as *Staphylococcus* for holotoxin [7], *Rotavirus* for diarrhea [36], dental caries caused by *S. mutans* [37], and *H. pylori* for gastric ulcers [4].

In this study, we tried to produce the specific IgY against human EV71. This enterovirus was first reported in the United States in 1974 [13] and has subsequently been reported worldwide [38,39]. In Taiwan, some severe cases of human EV71 are reported every year after the devastating EV71 outbreak of 1998 [15]. The seriousness of EV71 infection lies in its complications, which include hand-foot-and-mouth disease, herpangina, aseptic meningitis, poliomyelitis-like paralysis, and fatal encephalitis [40]. Most fatal cases occur in children under 3 years old, and high mortality is correlated with brainstem encephalitis [41].

The specific IgY against EV71 in the egg yolk increased after the third week after the first immunization, though there were obvious differences among individual hens. This variation in antibody production, based on the specific IgY production, might be the result of differences in individual immune responses [3]. The origin of antigen also plays an important role in the immune response, as different outer membrane proteins or fimbrial adhesions from the bacteria affected the rate of IgY production [5,9]. When the protein antigen contained whole bacteria, or when the virus achieved better specific antibody production, the *in vivo* neutralization effect of the resultant antibody was also better [7]. This study used the whole virus as the antigen source to obtain a better IgY titer. The total protein obtained from an egg yolk by the water dilution method is around 595 mg per egg. This protein contains few lipids and lipoproteins, and accounts for one-sixth of the total protein of a yolk [42]. In this study, the purity of IgY in WSF was approximately 30–40% (Table 1). This demonstrates that the water dilution method can easily separate the IgY from other egg yolk components, as described previously [4,34].

For the production of IgY in trials 1–8, the immunization dosage of antigen used was doubled and the immunization schedule was modified. A longer interval of 7 weeks was applied after the fourth immunization, and the last boost was given on week thirteen. Thereafter, the average specific-IgY against EV71 between 3 and 18 weeks increased to 3.33% of the total IgY, which is significantly higher than that of the pre-test which is only 1% of the total IgY ($P < 0.5$). However, whether or not this modified protocol is beneficial to the production of specific IgY remains to be clarified.

An analysis of the relationship between the concentration of specific IgY with the neutralization titer of the same sample revealed no significant correlation (Table 2). This indicated that the amount of specific IgY estimated by the ELISA method was not a good indicator of the neutralization titer of EV71 virus in this study. Previous studies on the effect of antibodies against Bacillus spores, botulinum pentavalent (ABCDE) toxoid and Anthrax vaccine also suggest that ELISA is not a reliable indicator for the neutralization titers [43,44].

The neutralization titer itself is a better indicator of antibody functionality. We determined the concentration of specific IgY by ELISA method using the cell lysate from the whole virus particle as the coating antigen. The binding between all viral proteins and specific IgY would be counted as part of the IgY concentration, whether or not the specific antibody was effective in virus neutralization. Kovacs-Nolan et al. [45] studied the epitopes of human rotavirus (HRV) by IgY and found that only three out of the five sequential neutralization epitopes on the Wa strain subunit protein VP8 were directly involved in HRV neutralization [46,47]. Our results indicate that the therapeutic function was significant when neutralization titer of the anti-EV71 IgY was 128 or higher (Table 2). However, further research is necessary to identify the antibodies directly involved in the neutralization of EV71.

This study includes two panels of animal tests for the EV71 challenge and IgY treatment. In trial 1, we challenged 1-day-old mice with a mouse-adapted EV71 strain MP4 by intraperitoneally administering a dosage of 10^5 pfu per mouse, and treated with specific IgY of neutralization titer 64. The results revealed no therapeutic effect. On the contrary, if the neutralization titer value of specific IgY was 128 or higher, the protective effects were significant (Table 2). Previous studies also show that this level of neutralization titer is effective for the serum IgG of mice [31,35]. Compared to the control group, the mice in specific IgY treated group not only had a lower mortality, but also had normal weight gains. Most of the mice in control group showed a weight loss 5 days after the viral challenge, and continued to lose body weight until they died. Less than 10% of the sick mice in the control group survived the artificial infection of EV71 at a dose of 10^5 pfu per mouse. The surviving mice gained some weight despite showing signs of illness. After reaching a body weight of 4.5–5.0 g, they recovered from the infection and returned to healthy status. However, their subsequent weight gains were only 60–70% of the mice in the specific IgY treated group (data not shown). Some surviving mice also exhibited persistent paralysis of the hind limb and remained paralyzed for the rest of their lives, just like some children infected by EV71 [47].

In trials 1–4, the IP injection time of specific IgY began from the second day after viral infection and lasted for three consecutive days, before the EV71 syndrome appeared. In practical situations, children infected by EV71 will probably only receive the IgG treatment after they exhibit the typical symptoms. To determine the critical timing of effective treatment, trials 5 and 6 applied specific IgY treatment one and two days later, respectively. Clearly, the starting time of specific IgY injection was essential in reducing the mortality of infected mice. If IgY was given on 4–6 dpi, at which point most mice exhibited the symptoms of hind limb paralysis, only 37% of infected mice survived. It has been reported that on the first day of EV71 infection, the virus is kept in the small intestine, and then spreads to other organs on 2 dpi. The virus attacks most organs on 3 dpi, including the brain stem, lung, spinal cord, and muscle [31,48]. If we started the specific IgY treatment on 2 dpi, which corresponds to the onset of clinical symptoms in children, then the therapeutic outcome was improved. In this case, the survival rate of the EV71-infected mice could be increased to 79%, highlighting the importance of the timing of specific IgY treatment. Before the on-set of clinical symptoms, specific IgY treatment has a better chance of achieving effective therapy.

Some animal studies use oral feeding of egg yolk powder directly, without any purification of specific antibodies from the yolk [10,49]. These studies prove the possibility of passive protection from the de novo form of yolk powder. In this study, 4 to 5 day-old mice were orally challenged with a viral dosage of 3×10^6 pfu per mouse. Challenged mice in the specific IgY-treated group showed a lower morbidity rate of 29% (12/41) than the control group, which showed a morbidity rate of 79% (38/48). This indicates that the orally fed specific IgY effectively neutralized the viral attack in the gastroenteric duct, thereby blocking the infection of virus in challenged mice. The neutralization function appeared to be effective no matter if it was given 1 h before or after the viral challenge. The mortality among specific IgY treated mice was 24% (10/41), which was significantly lower than that of the control group, at 69% (33/48). However, the effect of specific IgY declined for pups aged 5 to 6 days old with a body weight ranging from 3.0 to 3.5 g. Previous research also shows that the age of mice affects the infection results [50]. Mice more than 6 days old would not be infected by EV71 after artificial challenge, indicating that the immune system of mice at this age could provide suitable protection.

When mice are challenged orally or received the IgY through an oral passage, the virus and IgY pass through gastroenteric duct and be digested by gastric juice, reducing the viral infectivity of the IgY function. This was noticed by the lower morbidity after oral challenge with the same strain of EV71 and a less protective function of IgY among infected mice, compared to the results in trials 1–6, where an infection rate near 100% and a curing function more than 90% was obtained. The IgY specific to human rotavirus exhibits a passive protection against diarrhea in mice [51]. Although gastric juice had some influence on IgY, was still protective when a sufficient amount of IgY was provided.

Oral administration of spray dried yolk antibodies specific against Salmonella typhimurium or Salmonella dublin is effective in preventing Salmonella infections in calves [5]. IgY raised against ETEC antigen has also been proven effective in controlling the diarrhea of piglets by oral administration [49]. Nevertheless, because the 4 to 6 day-old mice pups in this study could not consume egg yolk, we applied a purified antibody from IgY instead. This purified IgY, derived from the water soluble fraction of egg yolk, was more accessible to the gastric juice and easily exposed to digestive enzymes. The activity of might be influenced by the change of structure and loss of bioactivity [5,7]. Based on the limitations of oral administration in suckling pups, a higher titer of IgY or a continuous treatment of IgY after viral challenge might achieve greater protection. This possibility is worthy of further research. The results of this study, however, indicate the potential of IgY in the prevention of EV71 infection in young children.

Several studies have been conducted to evaluate the stability of these antibodies. The IgY is not degraded during pasteurization at 60 °C [52] IgY was stable after 30 min in 60–65 °C, but was no longer active after 20 min in 80 °C [53]. SO IgY technology being new potential market applications in medicine, public health, veterinary medicine and food safety. A broader use of IgY technology could be applied as biological or diagnostic tool, nutraceutical or functional food development, oral-supplementation for prophylaxis, and as pathogen-specific antimicrobial agents for infectious disease control.

In conclusion, the ICR mice IP challenged with mouse-adapted strain MP4 of EV71 at a dose of 10^5 pfu per mouse induced an EV71 infection resulting in a high mortality rate. However, a survival rate of 98.3% (60/61) was achieved if the challenged mice were IP injected, 1–3 dpi for 3 consecutive days, with a purified IgY antibody with a neutralization titer of 128 or more. When the challenge was carried out orally with a dose of 3×10^6 pfu per mouse, the lower morbidity in IgY treated group proved that the protection could be achieved by the neutralization of virus in the gastroenteric duct, thereby reducing the mortality of challenged mice.

This is the first study to evaluate the effect of IgY treatment on EV71 infection, and our positive results indicate that further application in the prevention of EV71 is possible. In the form of an egg-yolk-added drink, yolk powder tablet, or capsule, it is can potentially be used to prevent the early infection of EV71.

References

[1] Gassmann M, Thommes P, Weiser T, Hubscher U. Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. Faseb J 1990;4:2528–32.
[2] Leslie GA, Clem LW. Phylogen of immunoglobulin structure and function. Immunoglobulins of the chicken. J Exp Med 1969;130:1337–52.
[3] Amaral JA, Tino DE, Franco M, Carneiro-Sampaio MM, Carbonare SB. Anti-enteropathogenic Escherichia coli immunoglobulin Y isolated from eggs laid by immunised Leghorn chickens. Res Vet Sci 2002;72:229–34.
[4] Shin JH, Yang M, Nam SW, Kim JT, Myung NH, Bang WG, Roe IH. Use of egg yolk-derived immunoglobulin as an alternative to antibiotic treatment for control of Helicobacter pylori infection. Clin Diagn Lab Immunol 2002;9:1061–6.
[5] Yokoyama H, Umeda K, Peralta RC, Hashi T, Icatlo FCJr, Kuroki M, Ikemori Y, Kodama Y. Oral passive immunization against experimental salmonellosis in mice using chicken egg yolk antibodies specific for Salmonella enteritidis and S. typhimurium. Vaccine 1998;16:388–93.
[6] Hatta H, Tsuda K, Akachi S, Kim M, Yamamoto T, Ebina T. Oral passive immunization effect of anti-human rotavirus IgY and its behavior against proteolytic enzymes. Biosci Biotechnol Biochem 1993;57:1077–81.
[7] Leclaire RD, Hunt RE, Bavari S. Protection against bacterial superantigen staphylococcal enterotoxin B by passive vaccination. Infect Immun 2002;70:2278–81.
[8] Hatta H, Tsuda K, Ozeki M, Kim M, Yamamoto T, Otake S, Hirasawa M, Katz J, Childers NK, Michalek SM. Passive immunization against dental plaque formation in humans: effect of a mouth rinse containing egg yolk antibodies (IgY) specific to Streptococcus mutans. Caries Res 1997;31:268–74.
[9] Lee SB, Mine Y, Stevenson RM. Effects of hen egg yolk immunoglobulin in passive protection of rainbow trout against Yersinia ruckeri. J Agric Food Chem 2000;48:110–5.
[10] Ikemori Y, Ohta M, Umeda K, Icatlo FCJR, kuroki M, Yokoyama H, Kodama Y. Passive protection of neonatal calves against bovine coronavirus-induced diarrhea by administration of egg yolk or colostrum antibody powder. Vet Microbiol 1997;58:105–11.
[11] Kweon CH, Kwon BJ, Woo SR, Kim JM, Woo GH, Son DH, Hur W, Lee YS. Immunoprophylactic effect of chicken egg yolk immunoglobulin (IgY) against porcine epidemic diarrhea virus (PEDV) in piglets. J Vet Med Sci 2000;62:961–4.
[12] King AMQ, Brown F, Christian P, Hovi T, Hyypiä T, Knowles NJ, Lemon SM, Minor PD, Palmenberg AC, Skern T, Stanway G. Picornaviridae in Virus Taxonomy. Seventh Report of the International Committee for the Taxonomy of Viruses. Academic Press, New-York, San Diego, 2000, pp. 657–673.
[13] Schmidt NJ, Lennette EH, Ho HH. An apparently new enterovirus isolated from patients with disease of the central nervous system. J Infect Dis 1974;129:304–9.
[14] Hsiung GD, Wang JR. Enterovirus infections with special reference to enterovirus 71. J Microbiol Immunol Infect 2000;33:1–8.
[15] Ho M, Chen ER, Hsu KH, Twu SJ, Chen KT, Tsai SF, Wang JR, Shih SR. An epidemic of enterovirus 71 infection in Taiwan. Taiwan Enterovirus Epidemic Working Group. N Engl J Med 1999;341:929–35.
[16] Lum LC, Wong KT, Lam SK, Chua KB, Goh AY, Lim WL, Ong BB, Paul G, Abubaker S, Lamber TM. Fatal enterovirus 71 encephalomyelitis. J Pediatr 1998;133:795–8.
[17] Komatsu H, Shimizu Y, Takeuchi Y, Ishiko H, Takada H. Outbreak of severe neurologic involvement associated with Enterovirus 71 infection. Pediatr Neurol 1999;20:17–23.
[18] Wang SM, Lei HY, Huang KJ, Wu JM, Wang JR, Yu CK, Su IJ, Liu CC. Pathogenesis of enterovirus 71 brainstem encephalitis in pediatric patients: roles of cytokines and cellular immune activation in patients with pulmonary edema. J Infect Dis 2003;188:564–70.
[19] Rotbart HA, Webster AD. Treatment of potentially life-threatening enterovirus infections with pleconaril. Clin Infect Dis 2001;32:228–35.
[20] Rozhon E, Cox S, Buontempo P, O'connell J, Slater W, De Martino J, Schwartz J, Miller G, Arnold E, Zhang A. SCH 38057: a picornavirus capsid-binding molecule with antiviral activity after the initial stage of viral uncoating. Antiviral Res 1993;21:15–35.
[21] Lin YC, Wu CN, Shih SR, Ho MS. Characterization of a Vero cell-adapted virulent strain of enterovirus 71 suitable for use as a vaccine candidate. Vaccine 2002;20:2485–93.
[22] Hayden FG, Andries K, Janssen PA. Safety and efficacy of intranasal pirodavir (R77975) in experimental rhinovirus infection. Antimicrob Agents Chemother 1992;36:727–32.
[23] Rozhon E, Cox S, Buontempo P, O'Connell J, Slater W, De Martino J, Schwartz J, Miller G, Arnold E, Zhang A. SCH 38057: a picornavirus capsid-binding molecule with antiviral activity after the initial stage of viral uncoating. Antiviral Res 1993;21:15–35.
[24] Rotbart HA, Webster AD. Treatment of potentially life-threatening enterovirus infections with pleconaril. Clin Infect Dis 2001;32:228–35.
[25] Starlin R, Reed N, Leeman B, Black J, Trulock E, Mundy LM. Acute flaccid paralysis syndrome associated with echovirus 19, managed with pleconaril and intravenous immunoglobulin. Clin Infect Dis 2001;33:730–2.
[26] Pevear DC, Tull TM, Seipel ME, Groarke JM. Activity of pleconaril against enteroviruses. Antimicrob Agents Chemother 1999;43:2109–15.
[27] Abzug MJ, Keyserling HL, Lee ML, Levin MJ, Rotbart HA. Neonatal enterovirus infection: virology, serology, and effects of intravenous immune globulin. Clin Infect Dis 1995;20:1201–6.
[28] Huang CC. Neurological complications of enterovirus 71 infection in children: lessons from this Taiwan epidemic. Acta Paediatr Taiwan 2001;42:5–7.
[29] Yu CK, Chen CC, Chen CL, Wang JR, Liu CC, Yan JJ, Su IJ. Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. J Biomed Sci 2000;7:523–8.
[30] Yan JJ, Su IJ, Chen PF, Liu CC, Yu CK, Wang JR. Complete genome analysis of enterovirus 71 isolated from an outbreak in Taiwan and rapid identification of enterovirus 71 and coxsackievirus A16 by RT-PCR. J Med Virol 2001;65:331–9.
[31] Wang YF, Chou CT, Lei HY, Liu CC, Wang SM, Yan JJ, Su IJ, Wang JR, Yeh TM, Chen SH, Yu CK. A mouse-adapted enterovirus 71 strain causes neurological disease in mice after oral infection. J Virol 2004;78:7916–24.
[32] Akita EM, Nakai S. Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic *E. coli* strain. J Immunol Methods 1993;160:207–14.
[33] Akita EM, Nakai S. Immunoglobulins from egg yolk: isolation and purification. J Food Science 1992;57:629–34.
[34] Lee EN, Sunwoo HH, Menninen K, Sim JS. In vitro studies of chicken egg yolk antibody (IgY) against Salmonella enteritidis and Salmonella typhimurium. Poult Sci 2002;81:632–41.
[35] Yu CK, Chen CC, Chen CL, Wang JR, Liu CC, Yan JJ, Su IJ. Neutralizing antibody provided protection against enterovirus type 71 lethal challenge in neonatal mice. J Biomed Sci 2000;7:523–8.
[36] Sarker SA, Casswall TH, Juneja LR, Hoq E, Hossain I, Fuchs GJ, Hammarstrom L. Randomized, placebo-controlled, clinical trial of hyperimmunized chicken egg yolk immunoglobulin in children with rotavirus diarrhea. J Pediatr Gastroenterol Nutr 2001;32:19–25.
[37] Smith DJ, King WF, Godiska R. Passive transfer of immunoglobulin Yantibody to Streptococcus mutans glucan binding protein B can confer protection against experimental dental caries. Infect Immun 2001;69:3135–42.
[38] Alexander JPJR, Baden L, Pallansch MA, Anderson LJ. Enterovirus 71 infections and neurologic disease-United States, 1977–1991. J Infect Dis 1994;169:905–8.
[39] Samuda GM, Chang WK, Yeung CY, Tang PS. Monoplegia caused by enterovirus 71: an outbreak in Hong Kong. Pediatr Infect Dis J 1987;6:206–8.
[40] Melnick JL. Enterovirus type 71 infections: a varied clinical pattern sometimes mimicking paralytic poliomyelitis. Rev Infect Dis 1984;6(Suppl 2):S387–390.
[41] Wang SM, Liu CC, Tseng HW, Wang JR, Huang CC, Chen YJ, Yang YJ, Lin SJ, Yeh TF. Clinical spectrum of enterovirus 71 infection in children in southern Taiwan, with an emphasis on neurological complications. Clin Infect Dis 1999;29:184–90.

[42] Sungino H, Nitoda T, Juneja LR. General chemical composition of hen eggs. In: Yammamoti T, Juneja LR, Hatta H, Kim M, editors. Hen eggs:Their basic and applied science. CRC Press; 1997.
[43] Reuveny S, White MD, Adar YY, Kafri Y, Altboum Z, Gozes Y, Kobiler D, Shafferman A, Velan B. Search for correlates of protective immunity conferred by anthrax vaccine. Infect Immun 2001;69:2888–93.
[44] Weiss S, Kobiler D, Levy H, Marcus H, Pass A, Rothschild N, Altboum Z. Immunological correlates for protection against intranasal challenge of Bacillus anthracis spores conferred by a protective antigen-based vaccine in rabbits. Infect Immun 2006;74:394–8.
[45] Kovacs NJ, Yoo D, Mine Y. Fine mapping of sequential neutralization epitopes on the subunit protein VP8 of human rotavirus. Biochem J 2003;376:269–75.
[46] He Y, Zhou Y, Wu H, Luo B, Chen J, Li W, Jiang S. Identification of immunodominant sites on the spike protein of severe acute respiratory syndrome (SARS) coronavirus: implication for developing SARS diagnostics and vaccines. J Immunol 2004;173:4050–7.
[47] Hayward JC, Gillespie SM, Kaplan KM, Packer R, Pallansch M, Plotkin S, Schonberger LB. Outbreak of poliomyelitis-like paralysis associated with enterovirus 71. Pediatr Infect Dis J 1989;8:611–6.
[48] Chen YC, Yu CK, Wang YF, Liu CC, Su IJ, Lei HY. A murine oral enterovirus 71 infection model with central nervous system involvement. J Gen Virol 2004;85:69–77.
[49] Marquardt RR, Jin LZ, Kim JW, Fang L, Frohlich AA. Passive protective effect of egg-yolk antibodies against enterotoxigenic Escherichia coli K88+ infection in neonatal and early-weaned piglets. FEMS Immunol Med Microbiol 1999;23:283–8.
[50] Ishimaru Y, Nakano S, Yamaoka K, Takami S. Outbreaks of hand, foot and mouth disease by enterovirus 71. High incidence of complication disorders of central nervous system. Arch Dis Child 1980;55:206–8.
[51] Yolken RH, Leister F, Wee SB, Miskuff R, Vonderfech TS. Antibodies to rotaviruses in chickens' eggs: a potential source of antiviral immunoglobulins suitable for human consumption. Pediatrics 1988;81:291–5.
[52] Hatta H, Tsuda K, Akachi S, Kim M, Yamamoto T. Productivity and some properties of egg yolk antibody (IgY) against human rotavirus compared withr abbit IgG. Biosci Biotechnol Biochem 1993;57:450–4.
[53] Horie K, Horie N, Abdou AM, Yang JO, Yun SS, Chun HN, Park CK, Kim M, Hatta H. Suppressive effect of functional drinking yogurt containing specific egg yolk immunoglobulin on Helicobacter pylori in humans. J Dairy Sci 2004;87:4073–9.