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RESEARCH ARTICLE

Over-Expression of Rab1 Gene during Infectious Bursal Disease Virus Infection in Layer Chicken

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ABSTRACT

Infectious bursal disease virus (IBDV) can cause immuno-suppression and morbidity in chickens and mainly replicate in the bursa of Fabricius, spleen, thymus and other lymphoid tissues. Rab1 gene is one of Rab GTPases and mainly deal with intracellular protein transport between endoplasmic reticulum (ER) and Golgi. In the present study, SPF layer chickens were artificially challenged with IBDV and the relationship between Rab1 protein gene expression and virus replication was explored by real-time PCR. The aim of the current study was to initially understand dynamic expression of Rab1 during the IBDV infection and offer basic data for further study of IBDV pathogenesis. The data showed that the content of Rab1 peaked on day 3 P.I. in infected group and the Rab1 gene levels were about 2.7 times those of the mocked-infected group. Likewise, the contents of VP2 of chickens in the infected group peaked on day 4 post-infection. After that, the VP2 gene levels slowly dropped until chickens were sacrificed. Moreover, there were two amino acid mutation sites were found on sites 61 and 159 in the infected groups and Lysine (K) and threonine (T) were mutated into glutamate (E) and alanine (A), respectively. The change of mRNA expression level of Rab1 gene from chick bursa post IBDV infection suggested that Rab1 might play a vital role during IBDV replication.

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INTRODUCTION

Infectious bursal disease virus (IBDV), one typical double-stranded RNA virus, often leads to high morbidity and mortality of chickens in an acute form or severe immuno-suppression or secondary infections, bringing large economic losses for farmers throughout the world once its outbreak (Muller *et al.*, 2003; Eterradossi and Saif, 2008). The genome consists of two segments and encodes five viral proteins, designated VP1-5. Among them, VP2 induces neutralizing antibodies to elicit protective immune responses. Many amino acid changes of different IBDV strains occured in the hyper-variable region of VP2 (Letzel *et al.*, 2007). Thus, the VP2 hyper-variable region is the main target for the molecular techniques used for IBDV detection and virus variation studies. The bursa of Fabricius (BF) is the target organ of IBDV replication and the virus titers peaked between 3 to 5 days in BF after IBDV infection (Wang *et al*., 2011).

Rab GTPases belongs to one part of the monomeric GTPases members and mainly play important role in intracellular protein transport between organelles (Delevoye and Goud, 2015). Rab GTPases, defined as small GTP-binding proteins, have more than 60 proteins based on their similar function, structure, and other properties (Stenmark and Olkkonen, 2001). The Rabs take part in vesicular membrane transport by both the exocytic and endocytic pathways, and could allow transport carriers or vesicles to participate in specific effectors in their active GTP-bound form to mediate vesicular transportation (Grosshans *et al*., 2006). They function by switching between GDP-bound and GTP-bound states that regulate interactions with other proteins known as effectors. Rab4 has been found to be involved in early endocytic vesicles and rapid recycling of transferring receptors and glycosphingolipids (Bananis *et al*., 2003; Grant and Donaldson, 2009). Late endocytic vesicles contain Rab7 while Rab11 located in the recycling

endosome, the TGN and specialized membranes of regulated secretory pathways (Calhoun *et al*., 1998) and offers vesicle trafficking pathway to the cell periphery for influenza virus RNPs between the nucleus and the pericentriolar recycling endosome (Amorim *et al*., 2011). There are two isoforms of Rab1 (Rab1a and Rab1b) and they share 92% amino acid sequence homology. The role of Rab1 in ER-to-Golgi trafficking was to recruit the tethering factor p115 into a cis-SNARE complex that programs coat protein II (COPII) vesicles budding form the ER for fusion with the Golgi (Allan *et al*., 2000) with the help of the cis-Golgi tethering protein GM130 (Moyer *et al*., 2001).

Up to date, more and more studies have been focused on the effects of Rab family on viral replication and release. Moreover, the birnaviral replication process in a complex structure is also paid attention on nowadays. Delgui *et al.* has proved that IBDV replication occurred on endosomal membrane compartments and the Golgi complex played a role in viral assembly (Delgui *et al*., 2013). In the current study, SPF chickens were artificially challenged with IBDV and the relationship between Rab1 protein gene expression and virus replication was explored. The main goal of the current study was to initially understand dynamic expression of Rab1 during the IBDV infection and offer basic data for further identification potential IBDV replication inhibition pathway.

MATERIALS AND METHODS

Experimental design and Sample collection: A classic IBDV strain BC6/85 was obtained from China Institute of Veterinary Drug Control (Beijing, China). One hundred 21-day-old specific pathogen free layer chickens were purchased from Merial Vital Laboratory Animal Technology Co., Ltd (Beijing, China) and fed in negative-pressure isolators. The chickens were randomly divided into two groups: one group (n = 55) was inoculated with the IBDV strain BC6/85 at a dose containing $10^{6.23}$ EID_{50}/0.1 mL each through ocular-nasal, another group was treated with 0.1 mL sterile PBS (pH=7.36) each (n = 45) as the mock-infected control group. At 0, 12, 24, 48, 72, 96, 120, 144, 168 and 192 hours post-infection (P.I.), four chickens were randomly taken from each group for sample preparation. At necropsy, the BF was quickly taken and then used for bursa index [bursa index=weight of bursa (mg)×1000/body weight (g)]. One part of BF was used for virus load, another portion for gene expression of Rab1. BF tissue from the mock-infected group was used as parallel controls. All animal experiments in the study were approved by the scientific ethical committee of Henan Institute of Science and Technology.

Quantification of IBDV and Rab1 in BF: Total RNA was extracted from BF with TRIzol reagent (Life technologies, NY, USA), following the manufacturer's protocol, and then DNA degradation of the total RNAs and first-strand cDNA synthesis PrimeScript^{TM} RT reagent kits with gDNA eraser (TakaRa, Dalian, China) according to manufacturer's instructions. VP2 gene was used to quantify IBDV virus load while a pair of primer was designed for detection of Rab1. *β*-actin was applied as internal standard. The primer pairs for VP2 gene, Rab1 and *β*-actin were shown in Table 1. Each sample was performed in triplicate with the above primers. The real-time PCR procedure was described briefly as follows: an initial incubation for 30 s at 95°C, 40 cycles of 5 s at 95°C, 20 s at 57°C, and 20 s at 72°C. The relative quantification of target genes was based on the fluorescence intensity (TakaRa, Dalian, China). The specificities of the PCR products were assessed by dissociation curve analyses. Each sample was repeated in triplicate. After PCR, the data were analysed with the PikoReal^{TM} 2.1 (Thermo Scientific, Waltham, USA) using the $2^{-\Delta\Delta CT}$ method. The viral genes were shown by relative quantification to the content of *β*-actin.

Deduced amino acid analysis by DNAMAN software of complete length of Rab1 following infection with IBDV: One part of bursa collected on day 4 P.I. was used for the deduced amino acid analysis. The processes for total RNA extraction and reverse transcription were the same with as above described and then PCR reactions were applied by primers of complete length of Rab1. The primer was: Forward 5'-CACGGACATGTCCAGCAT GAACC-3' and Reverse 5'-CAATCTCTGACCTTTGT GGAGACG G-3'. The PCR reaction production, 764 bp in length, was purified for sequencing by Sangon Biotech (Shanghai, China) and deduced amino acids analysis by DNAMAN software (LynnonBiosoft, USA).

Statistical analysis: All data were analyzed through the software package SPSS 13.0 for Windows. Results were displayed as mean ±standard deviation (SD). A two-tailed one-way ANOVA was applied for all statistical analysis.

RESULTS

Bursal index largely decreased post infection with IBDV: Layer chickens in the infected group displayed appetite distress and ruffled feathers after challenged with IBDV BC 6/85. Postmortem lesions included typical symptoms, such as muscle hemorrhages, white urate deposition in kidney and enlargement or hemorrhage in bursa. To check the effects of IBDV on bursa, bursa index was calculated from day 1 to 8 P.I. between the mock-infected control group and infected group (Fig.1). BF became atrophy and there were some exudates in BF. The bursa index largely decreased on day 1 (4.18) ($P<0.01$) and the trend remained the same until the end of the experiment on day 8 (1.19). However, bursa index of chickens in the mocked-infected group showed no significant changes and kept almost the same (from 3.8 to 4.5).

Table 1: Primers for Rab1, VP2 and *β*-actin used in the real-time PCR

Gene	Sense Primer	Anti-sense Primer	Fragment size (bp)
Rab1	5'-AGGAGATAGACCGTTATGCCAGTG-3'	5'-TCTACATTTGTGGCGTTCTTTGC-3'	166
VP2	5'-GGAGCCTTCTGATGCCAACAAC-3'	5'-CAGGAGCATCTGATCGAACTTGTAG-3'	215
β-actin	5'-TGTGCGTGACATCAAGGAGAAG-3'	5'-TACCACAGGACTCCATACCCAAG-3'	197

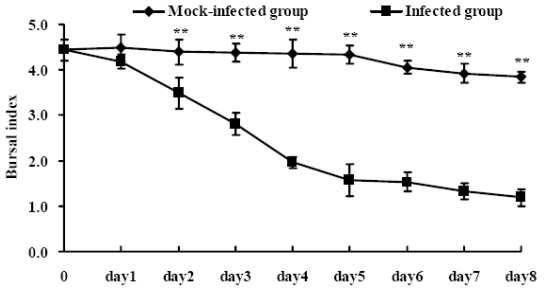

Fig.1: Bursal index changes post infection between the infected group and the mock-infected group. **Indicates bursa index in the mock-infected group were significantly higher than those of the infected group (P<0.01).

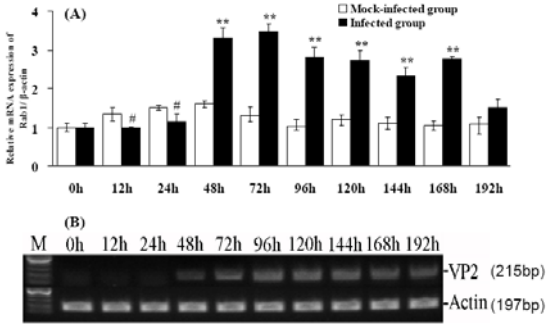

Fig. 2: Expression analysis of Rab1 post infection. (A) Relative fold change in transcriptional expression of Rab1 gene in BF was quantified after inoculation of IBDV and PBS. (B) VP2 gene levels of BF post infection and β-actin gene as internal control. The transcript level in mock-infected group (0 h) was normalized to 1, the ratios of to 0 h level in the following time were calculated. Values were shown as mean±SD (n=4). M: 100 bp ladder; Two asterisks indicated the very significant difference at P<0.01; # indicated the significant difference at P<0.05.

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                    *         20         *         40         *
Rab1       : MSSMNPEYDYLFKLLLIGDSGVGKSCLLLRFADDTYTESYISTIGVDFKIRTIELDGK :  58
Rab1 K61E  : MSSMNPEYDYLFKLLLIGDSGVGKSCLLLRFADDTYTESYISTIGVDFKIRTIELDGK :  58
Rab1 T159A : MSSMNPEYDYLFKLLLIGDSGVGKSCLLLRFADDTYTESYISTIGVDFKIRTIELDGK :  58
             MSSMNPEYDYLFKLLLIGDSGVGKSCLLLRFADDTYTESYISTIGVDFKIRTIELDGK

             60         *         80         *         100        *
Rab1       : TIKLQIWDTAGQERFRTITSSYYRGAHGIIVVYDVTDQESFNNVKQWLQEIDRYASEN : 116
Rab1 K61E  : TIELQIWDTAGQERFRTITSSYYRGAHGIIVVYDVTDQESFNNVKQWLQEIDRYASEN : 116
Rab1 T159A : TIKLQIWDTAGQERFRTITSSYYRGAHGIIVVYDVTDQESFNNVKQWLQEIDRYASEN : 116
             TI LQIWDTAGQERFRTITSSYYRGAHGIIVVYDVTDQESFNNVKQWLQEIDRYASEN
               △
             120        *         140        *         160        *
Rab1       : VNKLLVGNKCDLTTKKVVDYTTAKEFADSLGIPFLETSAKNATNVEQSFMTMAAEIKK : 174
Rab1 K61E  : VNKLLVGNKCDLTTKKVVDYTTAKEFADSLGIPFLETSAKNATNVEQSFMTMAAEIKK : 174
Rab1 T159A : VNKLLVGNKCDLTTKKVVDYTTAKEFADSLGIPFLETSAKNAANVEQSFMTMAAEIKK : 174
             VNKLLVGNKCDLTTKKVVDYTTAKEFADSLGIPFLETSAKNA NVEQSFMTMAAEIKK
                                                        △
               180        *         200
Rab1       : RMGPGATAGGAEKSNVKIQSTPVKQSSGGCC : 205
Rab1 K61E  : RMGPGATAGGAEKSNVKIQSTPVKQSSGGCC : 205
Rab1 T159A : RMGPGATAGGAEKSNVKIQSTPVKQSSGGCC : 205
             RMGPGATAGGAEKSNVKIQSTPVKQSSGGCC
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Fig. 3: Sequence alignment of the deduced amino acid of Rab1 between the infected group and the mock-infected group on day 4 post infection. The mutant sites were displayed with triangle at position 61 and 159.

The Rab1 and VP2 gene levels almost peaked at the same time: The mRNA transcript levels of Rab1 in chicken P.I. were quantified by real-time PCR and expression levels of genomic RNA copy number of VP2 were measured by semi-quantitative PCR and shown in Fig. 2. The Rab1 gene levels in bursa from chickens in the infected group contains a similar level at 12 h and 24 h to those in the mocked-infected group and dramatically increased (P<0.01) on day 2 compared to those of the mocked-infected group (Fig. 2A). The content of Rab1 peaked on day 3 P.I. in infected group and the Rab1 gene levels were about 2.7 times those of the mocked-infected group. Then the infected group slowly decreased until the end of the experiment except there was slightly increase on day 7. Likewise, the contents of VP2 of chickens in the infected group were quantified at corresponding time points and peaked on day 4 post-infection. After that, the VP2 gene levels slowly dropped until chickens were sacrificed (Fig. 2B).

Stable Rab1 amino acid variations in chickens challenged with IBDV: On day 4 P.I., the Rab1 gene was amplified from bursa in both groups and amino acid sequences were deduced by DNAMAN software (Fig. 3). Two amino acid variation sites were found in the infected groups and there were no variation sites in the mock-infected group. Lysine (K) and threonine (T) were mutated into glutamate (E) and alanine (A) on sites 61 and 159, respectively.

DISCUSSION

The Rab proteins, part of Ras superfamily of small GTPases, located to the cytosolic face of specific intracellular membranes, switch between two conformations and function as regulators of different steps in membrane transport pathways (Stenmark and Olkkonen, 2001). Structure proteins and non-structure proteins play a vital role in viral replication and the process mainly depends on the regulation of Rab family (Neuman *et al*., 2013; Patel and Roy, 2014; Wiltzer *et al*., 2014). IBDV is a very severe viral disease and causes immunosuppression in chickens accompanied with other pathogen infections. The genome of IBDV encodes five important proteins: an RNA-dependent RNA polymerase (VP1), two major structural proteins (VP2 and VP3), a viral protease (VP4) and a non-structural protein (VP5). The transportation of these proteins might be related with Rab family and site mutation is probably involved with virus replication (Qi *et al.*, 2013; Lai *et al.*, 2014).

IBDV BC6/85 is a classical Chinese virulent strain and can cause immunosuppression without or with little mortality (Wang *et al*., 2011). Bursa index in this study was consistent with previous study and these data showed the model was successful (Fig.1). In the current study, the IBDV infection model has shown that IBDV VP2 gene expression slowly increased P.I. accompanied with the growth of Rab1 gene expression in bursa collected from layer chickens in the infected group (Fig.2).

There are more than 60 human Rab and Rab-like members of the Ras super-family with many known functions (Hutagalung and Novick, 2011). Rab GTPases act as molecular switches and cycle between active and inactive states (Brumell and Scidmore, 2007) to function between intracellular signaling and membrane transport in a spatially and temporally sensitive manner (Bucci and Chiariello, 2006). In another words, Rab family will play an important role in the process of viral protein transportation and virus replication. Rab5 is needed for FMDV infection as expression of dominant-negative

Rab5 reduced the number of FMDV-infected cells by about 80% (Johns *et al*., 2009). Influenza virus ribonucleoproteins are routed from the nucleus to the pericentriolar recycling endosome through a Rab11-dependent vesicular transport pathway to the cell periphery (Amorim *et al*., 2011). Over-expressing 37 human Rab GTPase-activating proteins and determining virus titers displayed that Rab1a/b and Rab43 played important role in Herpes Simplex Virus 1 secondary envelopment (Zenner *et al*., 2011). Moreover, a *PjRab* gene was up-regulated in white spot syndrome virus-resistant shrimp, which indicated the Rab protein might take part in shrimp immune response to inhibit virus infection (Wu and Zhang, 2007) . Our data has shown that Rab1 gene level was also elevated in IBDV-infected chickens and two amino acids mutation sites were found during IBDV virus replication. Previous studies has shown that IBDV replication complexes were located to vesicular structures bearing features of early and late endocytic compartments by interfering with the endocytic pathway. Meanwhile, Golgi complex was also involved in the step of IBDV assembly (Delgui *et al*., 2013).

An interesting finding in this study was that there were two mutation sites in Rab1 gene, which might play a vital role in IBDV replication. Vesicular stomatitis virus glycoprotein was accumulated in many pre-*cis*-Golgi vesicles and vesicular-tubular clusters in the presence of a dominant-negative Rab1a mutant (Balch *et al*., 1994). A dominant-negative form of Rab11 family interacting protein 2 (FIP2) reduced the supernatant-associated respiratory syncytial virus titer 1000-fold and increased the cell-associated virus titer (Utley *et al*., 2008). Obviously, further study on the role of Rab1 gene mutations in the process of IBDV replication and pathogenicity should be taken consideration based on the current data and previous reports.

Conclusions: In this study, the current data displayed that Rab1 gene largely increased accompanied with VP2 gene of IBDV and Rab1 might be helpful for IBDV replication in bursa. Moreover, the two amino acid mutation sites on sites 61 and 159 might be involved in virus proliferation.

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Author's contribution: MJY and YY designed the experiment and analyzed the PCR data. ZHH and MHZ were helpful in animal experiment. OCB did the PCR, analyzed the final data and wrote the manuscript. WXN and LXY analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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