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Analysis of the Effect of Virulent Marek's Disease Virus *SORF2* Gene on its Horizontal Transmission Capacity Using Real-Time PCR

[1]Shuai Su, [1]Ning Cui, [1]Yixin Wang, [2]Aijun Sun, [1]Pengfei Qi, [1]Zimeng Chen,
[1]Peng Zhao and [1]Zhizhong Cui
[1]College of Veterinary Medicine, Shandong Agricultural University, Tai'an, 271018 Shandong, China
[2]Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences,
Texas A&M University, College Station, 77843 TX, United States

Abstract: Marek's Disease Virus (MDV) Chinese strain GX0101 was the first reported recombinant MDV field strain with one Reticuloendotheliosis Virus (REV) Long Terminal Repeat (LTR) insert. REV LTR in the GX0101 genome increased the potential for horizontal transmission. REV LTR fragment has the biggest impact on *SORF2* gene. Researchers detected the influence of *SORF2* gene deletion on its horizontal transmission ability by real time PCR. The result shows that GX0101ΔSorf2 possesses similar replication ability with bac-GX0101 no mater on the level of lymphocyte or feather follicle. Although, researchers can detect MDV positive in feather follicles of sentinel in contact chickens at age 10 in both groups respectively, the number of SPF chickens infected with bac-GX0101 by contact is higher than the number of GX0101ΔSorf2 infection group. Therefore, *SORF2* gene has certain effect on the horizontal transmission ability of the virus.

Key words: Marek's disease virus, *SORF2* gene, horizontal transmission capacity, real-time PCR, China

INTRODUCTION

Marek's Disease Virus (MDV) belongs to the family Herpesviridate, subfamily Alphaherpesvirinae. It causes Marek's disease in chickens with the development of lymphocytic infiltration of peripheral nerves, skin, skeletal muscle and visceral organs (Lampert *et al*., 1977; Witter, 1997). MDVs are classified into three serotypes: serotype 1 and serotype 2 as well as serotype 3 or Herpesvirus of Turkeys (HVT) (De Boer *et al*., 1986; Okazaki *et al*., 1970; Schat and Calnek, 1978). MDV serotype 1 viruses include the oncogenic MDVs which are grouped into three pathotypes ranging from mild (m) MDV up to very virulent plus (vv+) MDV strains. Its genome contains a linear double strand DNA of about 175 kb, encoding >100 open reading frames some of which are associated with the oncogenicity of the virus (Lee *et al*., 2000; Tulman *et al*., 2000). The *SORF2* gene, encoding a 179 aa protein is found only in serotype 1 (virulent) MDV strains, although it is not essential for tumor formation (Jang *et al*., 1998).

The MDV recombinant clone RM1 which was derived from the MDV strain JM/102W co-cultivating with Reticuloendotheliosis Virus (REV) has the integration of a REV Long Terminal Repeat (LTR) upstream of both *SORF1* and *SORF2* gene (Jones *et al*., 1993, 1996). In addition, there is a 3.2 kb transcriptis initiated from the LTR promoter overexpressed in RM1 which extended across the coding sequences of *SORF2* gene and two more downstream genes including *US1* and *US10* gene (Jones *et al*., 1996; Kim *et al*., 2011). Most interesting, the RM1 strain is attenuated for oncogenicity but retains contact spread (Liu *et al*., 2001). Besides, RM1 obtained another REV LTR in its TRS during the passage.

In contrast, MDV Chinese strain GX0101 was the first reported recombinant MDV field strain with one 538 bp REV LTR insert (Cui *et al*., 2010; Su *et al*., 2012; Zhang and Cui, 2005). GX0101 belongs to very virulent (vv) MDV but has higher horizontal transmission ability than vv MDV strain Md5. In the earlier studies, researchers cloned the GX0101 as an infectious Bacterial Artificial Chromosome (BAC) and carried out mutagenesis to delete the REV LTR to compare the horizontal transmission ability (Sun *et al*., 2010). Detection of the viral DNA in the feather follicle showed that the horizontal transmission of GX0101 virus deleting the REV LTR was delayed for a week. The results demonstrated that retention of the REV LTR in the MDV genome increased the potential for horizontal transmission. The difference between GX0101 and RM1 is the insertion location of the REV LTR in their genomes. GX0101 genome contains a solo REV LTR located at the site of 267 bp upstream to

Corresponding Author: Zhizhong Cui, College of Veterinary Medicine, Shandong Agricultural University, Tai'an, 271018 Shandong, China

SORF2 gene but within *SORF1* gene, it was 1 kb downstream compared to that of the RM1 strain (Su *et al*., 2012). The REV LTR is a strong promoter or enhancer and it may trans-activate different genes depending on the location of the insertion (Jones *et al*., 1996) such as expression of *SORF2* gene which is just downstream to the insertion site in GX0101.

In order to analyze the effect of the recombinant MDV field strain *GX0101SORF2* gene on its horizontal transmission capacity, researchers generated a mutant virus named GX0101ΔSorf2 lacking the solo *SORF2* gene, based on the GX0101 BAC clone. In this study, researchers used the GX0101 *meq* gene and chicken *ovotransferrin* gene as real-time PCR targets for the viral genome and the host cell genome, respectively. The duplex PCR measures the two targets in a single reaction. GX0101 BAC clone and a plasmid bearing a fragment of the *ovotransferrin* gene were used to produce standard curves. Using this method, researchers permitted quantitation of the MDV genome in cells derived from feather follicles to determine whether the SPF chicken infected MDV.

MATERIALS AND METHODS

Experimental chickens: SPF chickens were randomly divided into three equal groups (30 in each group) at 1 day of age and reared separately in isolators with positive filtered air. At 1 day of age, 15 chickens in group 1or 2 were inoculated intra-abdominally with 1000 PFU of bac-GX0101 or GX0101ΔSorf2 virus while the other 15 chickens with non-infected. Chickens in group 3 were inoculated with uninfected CEF cultures used as negative control.

Samples from MDV-infected and control chickens: Blood samples in anticoagulants were collected from six inoculated chickens of group1 and 2 on days 7, 14, 21, 28 post-infection, Peripheral Blood Lymphocytes (PBL) were prepared by centrifugation as earlier described (Baigent *et al*., 1996). Three to five pieces of feather follicles from each bird were collected for 4 weeks continuously post-infection.

DNA preparation from peripheral blood lymphocytes and feather follicles: DNA from peripheral blood lymphocytes and feather follicles were prepared by phenol chloroform extraction (Sambrook *et al*., 1989). The DNA was adjusted to 50 ng uL^{-1} in water and then stored at -20°C.

Absolute quantification of MDV genomes in test samples use real-time quantitative duplex PCR: Each reaction contained 0.5 uM of each primer and 0.2 uM of the corresponding probe synthesized by Shanghai Genecore Biotechnologies Co., Ltd. (Table 1), 10 uL of TaqMan® Gene Expression Master Mix (Applied Biosystems), 2 uL of DNA template in a total reaction volume of 20 uL. There were duplicate reactions for each sample.

An ABI PRISM® 7500 sequence detection system (Applied Biosystems) was used to amplify and detect the reaction products under the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles consisting of denaturation at 94°C for 15 sec and annealing/extension at 60°C for 1 min.

The results of real-time quantitative duplex PCR assays were analyzed using ABI 7500 Software Version 2.0.1 supplied with the ABI PRISM® 7500 sequence detection machine (Applied Biosystems). The default settings of the program were used to define both the threshold value and baseline for analysis of the raw data. A standard curve for each primer set was generated in each assay and used to derive the copy number of target sequences in test samples.

Preparation of constructs for producing standard curves: GX0101-BAC is a stable infectious BAC clone of the whole genome of the GX0101 (Sun *et al*., 2009). It was used as a standard for calibration of bac-GX0101 or GX0101ΔSorf2 genome copy number by detection of the region of *meq* gene. For calibration of the host genome copy number of chicken, pMD18-ovo prepared by cloning the chicken *ovo* gene (Gene Bank No.Y00407.1. pMD18-ovo-F: 5'-CAGCTCT AGCCAAA GCAATT-3'; pMD18-ovo-R: 5'-TTCAACCTTGTTGTCATCCC-3') into the T-tagged site of pMD18-T Easy vector (TaKaRa) was used as a standard. The concentration of GX0101-BAC and pMD18-ovo DNA was determined using the mean of

Table 1: Oligonucleotide primers and probes used in real-time duplex PCR

Target sequence	Primer/probe name	Primer sequence (5'-3')	Amplicon size (bp)
GX0101 *meq* gene (Su *et al*., 2012)	Meq forward	GAGCCGGAGAGGCTTTATGC	73
	Meq reverse	TATAAATCTGGCCCGAATACAA	
	Meq probe	GTCTTACCGAGGATCCCGAACAGG (5'-FAM label, 3'-TAMRA label)	
SPF chicken *ovotransferrin* gene (Jeltsch *et al*., 1987)	Ovo forward	CACTGCCACTGGGCTCTGT	71
	Ovo reverse	GCAATGGCAATAAACCTCCAA	
	Ovo probe	AGTCTGGAGAAGTCTGTGCAGCCTCCA (5'-Hex label, 3'-TAMRA label)	

five aliquots of two fold dilutions by spectrophotometry and the number of molecules of each cloned plasmid per microliter was determined. Master stock containing 10^9 copies of GX0101-BAC and 10^9 copies pMD18-ovo per 2 uL were used to prepare a 10 fold dilution series to detect the lower limit of detection of each assay.

Statistical analysis: Statistical analysis was performed with the Statistical Program for Social Sciences (SPSS) (SPSS Inc., Chicago, IL, USA). A $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Assay efficiency and reproducibility: Triplicate dilution series were run for GX0101-BAC and pMD18-ovo together or alone and copy number plotted against mean Cycle threshold (Ct) value. Reactions were specific for the meq gene and the *ovo* gene, respectively. The presence of the pMD18-ovo template had no significant effect on amplification of the GX0101-BAC template and vice versa. Reactions progressed with similar efficiency (Table 2).

Intra-assay variation was determined from triplicate duplex assays (using the mixture of GX0101-BAC and pMD18-ovo templates) within one PCR run. Inter-assay variation was determined using independently prepared dilution series run in triplicate within two further PCR runs on different days. Figure 1 shows mean Ct values from the three assays with 95% confidence limits for the regression.

Specificity and validation of real-time quantitative duplex PCR: To detect the specificity of the *meq* gene probe, five chickens were taken as samples from each group, DNA was extracted from feather follicles for the amplification of *ovo/meq* genes by real-time quantitative duplex PCR. The meq q-PCR was completely specific for the MDV virus genome while no background signal within samples from untreated chickens (Fig. 2a). The meq and ovo standard curves were used to calculate MDV genome copy No. per 10^4 cells for replicate assay of each feather follicles sample and mean virus genome copy No. for MDV virus was determined (Fig. 2b). Based on the specificity, reproducibility and validity of the meq and ovo reactions, these assays were used to investigate replication of MDV viruses in feather tips samples taken from chickens.

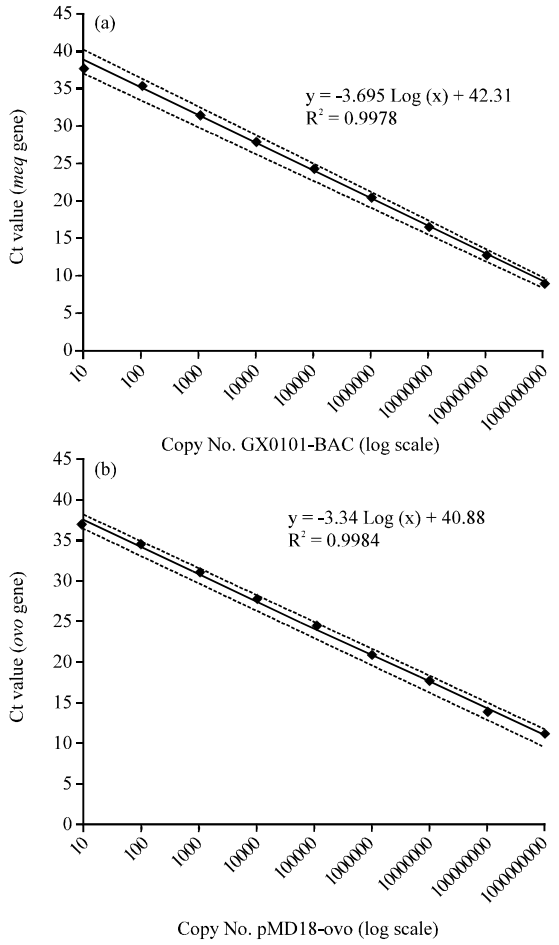

Fig. 1: Reproducibility of standard curves. The reproducibility of standard curves for the meq reaction; a) and the ovo reaction b) was determined using data from reactions performed in triplicate in three PCR runs on different days, using independently prepared dilution series of standard constructs GX0101-BAC and pMD18-ovo. The solid line, obtained by linear regression analysis, indicates the mean Ct values from the three assays and the dotted lines indicate the 95% confidence limits for the regression

Table 2: Optimisation of duplex real-time PCR standard reactions

		meq standard curve			ovo standard curve		
Primers	Standard construct (s) used as template	Slope	Y-axis intercept	R^2-value[a]	Slope	Y-axis intercept	R^2-value[a]
meq+ovo	GX0101-BAC	-3.682	42.29	0.998	----------No amplification[b]----------		
meq+ovo	pMD18-ovo	----------No amplification[b]----------			-3.37	41.01	0.999
meq+ovo	GX0101-BAC+pMD18-ovo	-3.707	42.32	0.998	-3.31	40.75	0.998

[a]R^2 coefficient of regression. [b]There was no amplification of the meq gene in the absence of the GX0101-BAC template and no amplification of the *ovo* gene in the absence of the pMD18-ovo

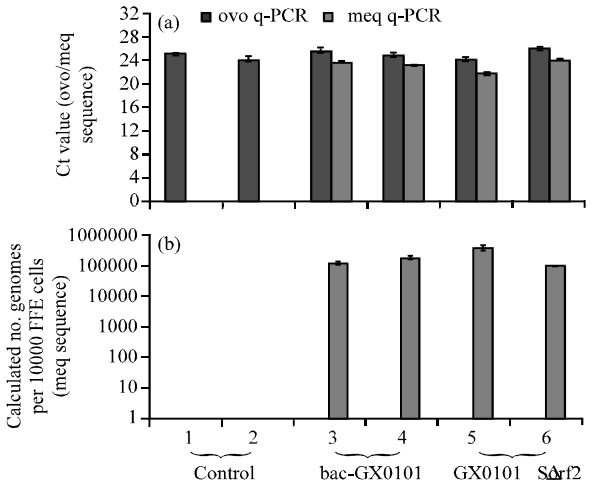

Fig. 2: a) Specificity of the meq and ovo reactions was examined using DNA from feather follicles of untreated chickens or chickens which had been challenged with bac-GX0101or GX0101ΔSorf2 as indicated under the histograms; b) Calculated virus genome copy No. per 10^4 FFE cells (log scale) for virus measured by meq assay

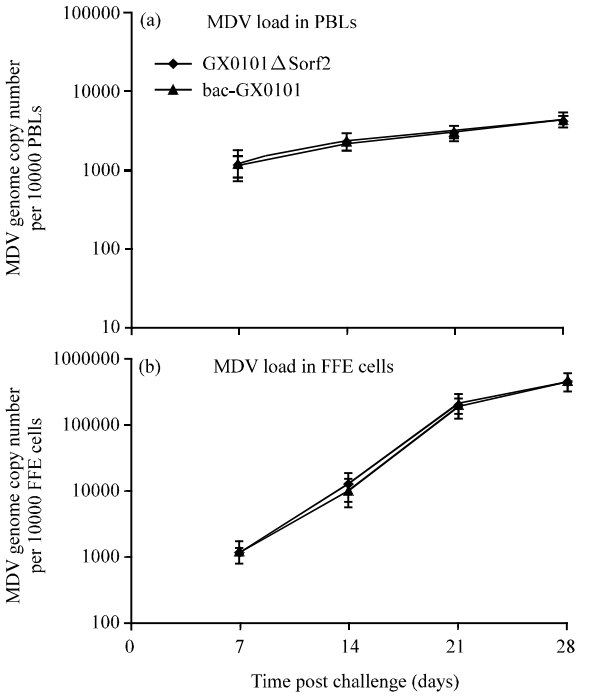

Fig. 3: Comparison of the *in vivo* replication kinetics of bac-GX0101 and GX0101ΔSorf2 viruses as mean genome copies per 10^4 a) PBLs and b) FFE cells from groups of six chickens plotted on a logarithmic scale

Growth kinetics of GX0101ΔSorf2 *in vivo*: The replication kinetics of both viruses was analyzed at various times in DNA samples prepared from PBL and the feather follicles using duplex real-time qPCR test. MDV genome copy numbers in the PBL from chickens infected with the two viruses were maintained at similar levels (~10^3 per 10^4 PBLs) after the initial sampling period of 7 days post-infection (p.i.) (Fig. 3a). DNA samples prepared from feather follicles showed a significantly higher MDV genome copy number (10^4 per 10^4 FFE cells) from the second sampling period of 14 days p.i., relative to the copy number in the PBL (Fig. 3b).

Horizontal transmission ability of bac-GX0101 and GX0101ΔSorf2: GX0101 virus reconstituted from the BAC clone retained its ability for horizontal transmission. This allowed us to examine the ability of the GX0101ΔSorf2 virus for chicken to chicken transmission. In SPF chickens challenged with GX0101ΔSorf2 or bac-GX0101 viruses at 1 day of age, MDV DNA could be detected in feather follicles by real-time quantitative duplex PCR 7 days after challenge. Furthermore, MDV DNA was also detected in feather follicles of sentinel in contact chickens at age 10 in both groups. The frequency of horizontal transmission was higher for bac-GX0101 than for GX0101ΔSorf2 virus, the difference was not statistically significant before 14 days of age ($p>0.05$) but statistically significant after 21 days of age ($p<0.05$) (Table 3). The slightly reduced transmission capacity of GX0101ΔSorf2 virus suggested that the *SORF2* gene in bac-GX0101 genome might contribute to virus horizontal transmission.

Various approaches have been used to detect and quantify MDV including virus isolation preformed in chick kidney cells (Churchill and Biggs, 1967) and detection of the MDV genome by in situ hybridization (Holland *et al*., 1996) or by PCR amplification. In recent years, real-time quantitative PCR was widely used to quantify copy numbers of the MDV genome with the advantages of rapid, sensitive, reproducible and has a wide dynamic range (Baigent *et al*., 2005a, b; Islam *et al*., 2006; Haq *et al*., 2012; Renz *et al*., 2013). As for the study on kinetics of MDV, absolute quantitation of viral load is far more meaningful than relative quantitation. Therefore, researchers used the *meq* gene and the chicken ovotransferrin (*ovo*) gene as PCR targets for the MDV (bac-GX0101 and GX0101ΔSorf2) genome and the host cell genome, respectively. GX0101-BAC was used as a standard for calibration of bac-GX0101 or GX0101ΔSorf2 genome copy number by detection *meq* gene. Different from the traditional meq-plasmid standard, copy number of GX0101-BAC directly relates to copy number of the

Table 3: Dynamics of different MDV strains detected from feather follicles of MDV-infected or uninfected chickens

	Number (%) of feather follicle samples positive for MDV genomic DNA									
	Replicate 1 (day)					Replicate 2 (days)				
Virus strain	7	10	14	21	28	7	10	14	21	28
bac-GX0101										
Challenged	7/15(47)	10/15(67)	13/15(87)	15/15(100)	13/13(100)	8/15(53)	9/15(60)	13/15(87)	15/15(100)	13/13(100)
In contact	0/15(0)[a]	2/15(13)[a]	4/15(27)[a]	10/15(67)[a]	13/15(87)[a]	0/15(0)[a]	3/15(20)[a]	4/15(27)[a]	11/15(73)[a]	13/15(87)[a]
GX0101ΔSorf2										
Challenged	6/15(40)	10/15(67)	12/15(80)	15/15(100)	14/14(100)	6/15(40)	9/15(60)	12/15(80)	15/15(100)	13/13(100)
In contact	0/15(0)[a]	1/15(7)[a]	2/15(13)[a]	4/15(27)[b]	7/15(47)[b]	0/15(0)[a]	2/15(13)[a]	2/15(13)[a]	5/15(33)[b]	6/15(40)[b]
Control										
Non-challenged	0/30(0)	0/30(0)	0/30(0)	0/30(0)	0/30(0)	0/30(0)	0/30(0)	0/30(0)	0/30(0)	0/30(0)

At 1 day of age, 15 chickens were inoculated intra-abdominally with 1000 PFU of bac-GX0101 or GX0101ΔSorf2 virus while sentinel in contact chickens with non-infected. Different letters (shown as superscript) indicate that the differences were statistically significant between groups ($p<0.05$)

MDV genome, since both contain two copies of the *meq* gene (Kaiser *et al*., 2003). To quantify virus genomes per cell, researchers used the pMD18-ovo plasmid containing part of chicken *ovo* gene to produce a standard curve (Fig. 1). The *ovo* gene is a house-keeping gene presents twice in each cell which can thus be used to normalize each sample. The presence of the pMD18-ovo template had no significant effect on amplification of the GX0101-BAC template and vice versa (Table 2). Hence, the duplex PCR measures the virus *meq* gene and the chicken *ovo* gene can in a single reaction (Fig. 2). Therefore, researchers can carry out absolute quantification for MDV genome copies by means of detecting MDV GX0101 strain *meq* gene and chicken host cell *ovo* gene as compared with previous relative quantification for MDV genome copies. The results are more comparable. In comparison with Baigent *et al.* (2005b), this study produced standard curve for viruses using infectious clone GX0101-BAC of vv MDV GX0101 whole genome. This also directly relates to copy number of the GX0101 or GX0101ΔSorf2 genome with slightly higher sensitivity (limit of detection 4 copies for the *meq* gene and 1 copy for the *ovo* gene). Further, any *MDV* gene in addition to *meq* gene for which a primer/probe set is available could be used as a target for the q-PCR to display the virus copy number.

Marek's Disease Virus (MDV) Chinese strain GX0101 was a recombinant MDV field strain with one Reticuloendotheliosis Virus (REV) Long Terminal Repeat (LTR) insert. The primary experiments demonstrated that retention of the REV LTR in the MDV genome increased the potential for horizontal transmission (Sun *et al*., 2009, 2010). The REV LTR, as a strong promoter or enhancer, affected transcription of the MDV *SORF2* gene and probably induce an enhanced expression of MDV SORF2 protein (Jones *et al*., 1996; Kim *et al*., 2011). So, in order to analyze the effect of GX0101 *SORF2* gene following REV LTR on its horizontal transmission capacity, researchers constructed a *SORF2* gene null virus, GX0101ΔSorf2 in which the solo *SORF2* gene had been deleted on the basis of GX0101 infectious cloning GX0101-BAC. Detected by the established dual real time PCR method, GX0101ΔSorf2 has the same multiplication rate as bac-GX0101 and gene copy number in chicken feather follicles is much higher than in lymphocytes (Fig. 3). Therefore, it does not affect the proliferation of MDV in chickens after the deletion of SORF2, demonstrating that SORF2 is not essential for the replication of MDV.

Although, GX0101ΔSorf2 has the same multiplication level as bac-GX0101 in feather follicles and MDV can be detected from DNA of feather follicles 7 days post inoculation respectively, the number of MDV positive chickens by contact infection differs and it differs significantly 21 days post inoculation (Table 3). That is to say, SORF2 has a certain influence on horizontal transmission capacity of MDV. Combined with previous results, it can be confirmed that the increased expression of SORF2 improved the horizontal transmission capacity of MDV GX0101 owning to the REV LTR insertion, making it the epidemic strain in the lower restructuring events.

CONCLUSION

In this study, researchers developed a method for absolute quantification of MDV copy numbers exactly which can be used to determine whether MDV was infected. Using this method, researchers detected that the horizontal transmission capacity of the SORF2 deleted virus appeared to be weaker than its parental GX0101 virus. These results provide experimental evidence for further research of recombinant MDV GX0101 strain becoming popular strain.

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