ABSOLUTE QUANTIFICATION OF MAREK'S DISEASE VIRUS SEROTYPE 2 (MDV2) USING REAL-TIME POLYMERASE CHAIN REACTION AND ITS APPLICATION TO FIELD DUST SAMPLES

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Summary

Methods for taqman real-time PCR assays to detect the three serotypes of MDV are available (Islam *et al.,* 2004), and an absolute quantification has been developed for MDV serotype 1 and serotype 3. The development of a method for absolute quantification of Marek's disease virus serotype 2 (MDV2) is described in this paper. Thus, it is now possible to perform qPCR assays for all three serotypes of MDV on a sample. Absolute quantification of MDV2 in dust samples from poultry farms across Australia in a preliminary study, revealed the presence of MDV2 in 13 of 30 samples tested.

I. INTRODUCTION

Marek's disease virus (MDV), an avian alphaherpesvirus, is one of the most potent oncogenic herpesviruses and causes a contagious, lymphoproliferative disease in chickens. MDV strains are classified into three serotypes based on their pathogenicity. Serotype 2 of MDV is a naturally occurring, infectious virus in chickens, but is nonpathogenic or only weakly pathogenic and nononcogenic in chickens (Baigent and Davison, 2004). The nononcogenic turkey herpesviruses (also referred to as HVT) are classified as serotype 3 viruses. Several strains of MDV2 are used as vaccines, either alone or combined with either strains of serotypes 1 or 3 of MDV. Immunisation with vaccinal strains of MDV does not prevent productive infection by MDV1, so multiple strains can co-exist in the host.

Since the introduction of polymerase chain reaction (PCR) in 1980, several specific assays have been developed and used to detect and quantify MDV (Zelnik, 2004). Real-time quantitative PCR (qPCR) provides a tool for the rapid detection and quantification of MDV and is increasingly preferred to conventional PCR which is labour intensive and requires post-PCR handling (Niesters, 2001). Real-time PCR assays to detect the three serotypes have previously developed by our group (Islam *et al.,* 2004), followed by methods for absolute quantification of MDV serotype 1 and serotype 3 (Islam *et al.,* 2005). In this paper we report the development and validation of a method for absolute quantification of MDV2 enabling the determination of MDV2 virus genome copy number in samples. With these methods we can now measure the viral copy number of all three serotypes in a single sample.

II. MATERIAL AND METHODS

MDV2 specific plasmid standards were developed using part of the sequence of the unique long region UL30 DNA*pol* gene containing qPCR primers described by Islam *et al.* (2004). A 283 bp fragment was amplified by standard PCR, using the reaction conditions described by Islam *et al.* (2005). Primers to produce this fragment were designed using Beacon designer 4.00 (PREMIER Biosoft International, Palo Alto, USA).

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The PCR products were purified using the Wizard® DNA purification Kit (Promega, Madison, USA) and ligated into the T-tagged site of the pGEM T-easy vector according to the manufacturer's protocol (Promega, Madison, USA). The ligation mix was transformed into competent E. *coli* (DH5α) and grown overnight on agar plates containing ampicillin/IPTG and X-gal. Positive transformants were identified using blue-white screening. DNA sequencing of the recombinant plasmid, designated pKR-DNA*pol*, was carried out by Newcastle DNA, University of Newcastle, Australia. Plasmid DNA was extracted and purified on large scale using a Wizard®plus Maxiprep DNA purification Kit (Promega, Madison, USA). The concentration of plasmid DNA was calculated on the basis of two identical agarose gel electrophoreses using twofold, fourfold and eightfold dilutions against a lambda standard containing a known amount of DNA. Purified plasmid DNA was stored at -20° C.

To establish the threshold of detection and demonstrate parallelism with the reference standard curve based on relative abundance, a series of tenfold dilutions, starting at $1x\ 10^6$ down to $1x\ 10^0$ plasmid molecules per reaction were made and a taqman real-time qPCR assay performed using a RotorGene 3000 real-time PCR machine (Corbett Research, Sydney, Australia). The qPCR assay, primer/probes set and reference standard curve used was set up as described by Islam *et al.* (2005). The reference standard curve comprised a 10-fold dilution series of DNA extracted from a cell-associated Maravac® vaccine (MDV2 strain MD19). To determine the reproducibility, three assays were run and intra- and inter-assay coefficients of variation calculated for both Ct value and calculated plasmid copy number. The assays were performed on separate days and all dilution series were made up new for each assay. To test the assay on a limited number of field samples, 30 dust samples from broiler farms around Australia were assayed for their MDV2 content.

III. RESULTS

a) Development of the method for absolute quantification of MDV2

The 283 bp fragment of the MDV2 specific UL30 DNA*pol* gene included the specific sequences of the qPCR primers described by Islam *et al.* (2004). These primer/probe sets do not cross-react when used in qPCR for the three serotypes (Islam *et al.,* 2004).

The fragment was cloned into the pGEM–T-easy vector (Promega Corporation) to produce the recombinant plasmid pKR-DNA*pol*. DNA sequencing of purified plasmid DNA confirmed that it contained the correct insert. Tenfold serial dilutions were made and amplification plots for the pKR-DNA*pol* plasmid standards derived from three separate assays are shown in Figure 1. The lowest dilution in the tenfold dilution series which amplified reliably was defined as the detection limit which was 10 copies of pKR-DNA*pol* plasmid. Standard curves were highly reproducible with no significant difference in slopes ($p<0.05$) between individual runs of the same assay. Based on three individual assays, the MDV2 assays showed mean intra-assay Ct- values with a CV value below 1%, while the mean inter-assay Ct- values had a CV below 3%. Calculated plasmid copy number had mean intra- and inter-assay CVs below 21.5%. Figure 2 shows a linear regression plot of pKR-DNA*pol* plasmid copy number against the previous MDV2 standard which is derived from Maravac® vaccine.

b) Absolute quantification of MDV2 genome in field dust samples

Out of the thirty samples taken from broiler farms across Australia which have been assayed previously for MDV1 and HVT, 13 samples amplified for MDV2 as shown in Table 1.

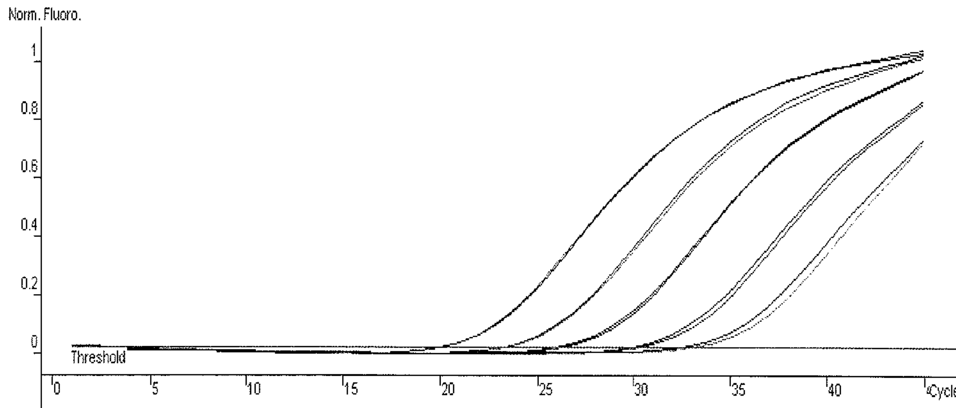

Figure 1: MDV2 assay. Amplification plot of DNA*pol* gene showing a serial tenfold dilution in duplicates of pKR-DNA*pol* plasmid copies (100000- 10), from left to right).

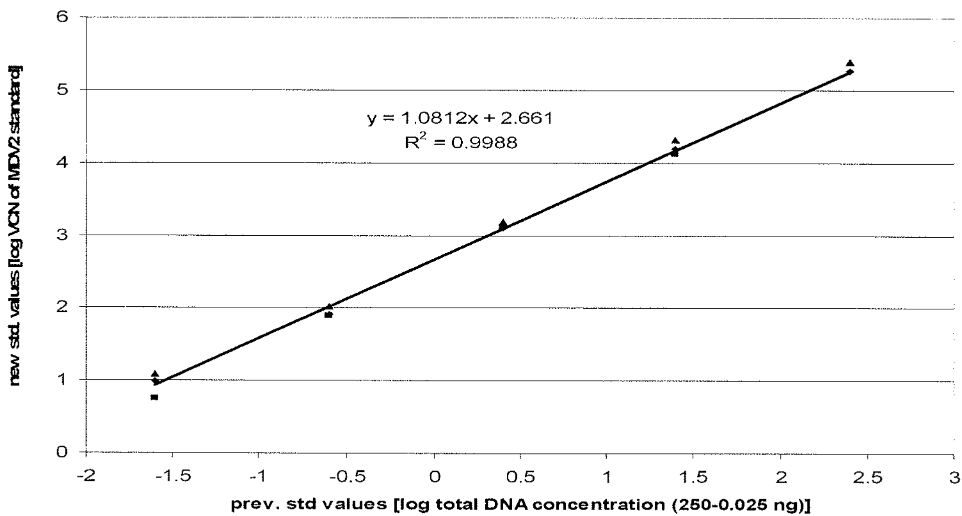

Figure 2: Conversion equation for conversion of previous standards to fully quantified standards, based on three assays.

Table 1: qPCR analysis of 30 field dust samples grouped by results for all three MDV serotypes

No. of samples	HVT vacc.	MDV1 assay	HVT assay	MDV2 assay	MDV2 VCN/mg dust [mean]
3	+	-	-	-	0
6	+	-	+	-	0
2	+	-	+	+	24054
2	+	+	+	+	8342
5	-	-	-	-	0
2	-	-	+	-	0
1	-	+	-	-	0
3	-	-	N.A.	+	22933
5	-	+	-	+	30469
1	-	+	+	+	2263
Total 30	13	9	13	13	

IV. DISCUSSION

The assay for the absolute quantification of MDV2 genome copy number in qPCR using the MDV2 specific plasmid, pKR-DNA*pol*, shows good reproducibility and the detection limit, defined to be the lowest dilution in the tenfold dilution series which amplified reliably, was 10 copies of pKR-DNA*pol* plasmid per reaction. As the lower detection limits were determined in a 10-fold dilution series, these values are regarded as conservative estimates. Therefore, the true detection limit lies in between this value and the next lower 1:10 dilution which did not amplify. The sensitivity of this MDV2 assay is greater than the sensitivity of the absolute quantification assay for HVT, reported by Islam *et al.* (2005), which amplified reliably 75 plasmid copies per reaction and is similar to that of the MDV1 assay reported by Islam *et al.* (2005) as 5 plasmid copies per reaction. One copy of the DNA*pol* gene represents one copy of the MDV genome as the DNA*pol* gene is present only in the unique long region. The generated plasmid standard curve showed parallelism with previous standards used (derived from dilutions of total DNA from 0.025–250 ng per reaction) from Maravac® vaccine indicating the same behaviour over a range from 10^3–10^5 dilutions. This data enabled us to convert the previous standard into viral copy number, thus allowing absolute quantification of qPCR assays.

From 30 field dust samples assayed to analyse the MDV2 viral load 13 samples amplified above the detection limit thus confirming its application in field samples containing a mixture of viruses. It is of interest that MDV2 was detected in 43% of dust samples from broiler farms where vaccination with MDV2 is not practiced. This suggests that wild type MDV2 is circulating in Australian broiler farms and warrants further detailed investigation.

To summarize, the application of this assay together with the MDV1 and HVT assays reported previously by Islam *et al.* (2005) enables us to absolutely quantify samples for all three serotypes of MDV. Furthermore, it is now possible to compare samples on the absolute quantification level. This should lead to improved understanding of the pathogenesis, spread, diagnosis and vaccinal control of Marek's disease.

ACKNOWLEDGEMENTS

This work was funded by the Australian Research Council project LP 0211607 for which we are grateful. We would like to thank Ms Megan Sutherland, Mrs Jenifer Druitt, Mr Suresh Kumar, Ms Sue Burgess and Mr Paul Reynolds for their help, provision of advice and technical support.

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