Assessment of the Diagnostic Ability of the DIVA Real-Time PCR in a Duplex Configuration to Differentiate Between the Turkey Meningoencephalitis Vaccine and Wild-Type Viruses

Davidson, I.* and Shimshon, Y.

Division of Avian Diseases, Kimron Veterinary Institute, Bet Dagan, Israel.

* **Corresponding Author:** Dr. Irit Davidson. Division of Avian Diseases, Kimron Veterinary Institute, Bet Dagan, Israel. Tel: 972-3-9681602; davidsonirit@gmail.com

ABSTRACT

The avian flavivirus *Turkey Meningoencephalitis Virus* (TMEV) causes a neuroparalytic disease of commercial turkeys, expressed in paresis, incoordination, drooping wings and mortality that can be controlled by vaccination. The newly developed Differentiating Infected from Vaccinated Animals Assay (DIVA), was further developed and transformed into a duplex assay aimed to identify in one amplification the virus type involved in the clinical signs, and to distinguish between wild-type TMEV strains and the vaccine virus. The performance of the newly developed multiplex DIVA assay was evaluated on the recent outbreak that affected most of the commercial turkey flocks. Using the TMEV vaccine virus we showed that the amplification parameters of the single-and duplex DIVA real-time PCR were similar. Next, the clinical cases were similarly amplified with the TMEV-DIVA assay both as single-and duplex DIVA real-time PCR. In conclusion, a powerful, distinctive, sustainable and sensitive assay was put to use during the 2017 TMEV outbreak.

Key words: Avian Flaviviruses; Turkey Meningoencephalitis Virus; Real-Time PCR; DIVA; Duplex; Outbreak.

INTRODUCTION

The *Turkey Meningoencephalitis Virus* (TMEV), causes a neuroparalytic disease in adult turkeys leading to paresis, incoordination, drooping wings and mortality due to inability to reach food and water (1). Vaccination of adult turkeys with a live-attenuated vaccine virus is practiced (1, 2), but occasionally typical clinical signs are noticed, causing uncertainty in diagnosis. Whether faulty vaccination or poor vaccine efficacy might be the cause, a differential diagnosis of the causative virus agent might supplement the diagnosis of affected commercial turkey flocks.

TMEV is an enveloped flavivirus, containing an 11 kb single-stranded positive-sense RNA genome, encoding for 3 structural (capsid (C), pre-membrane (prM) and envelope (*env*)) and 7 non-structural (NS) genes. The whole genome sequencing of 5 TMEV strains (3) facilitated the recent development of a TMEV molecular diagnosis assay aimed to Differentiate Infected from Vaccinated Animals (DIVA). Development of DIVA assays for molecular identification are relatively rare. Our development of the DIVA real-time RT-PCR (rtRTPCR) reflects further progress of the previous assay, where two distinct amplifications were aimed to detect differentially the TMEV vaccine and field viruses (5). In this study we aimed to provide an evolved and robust one-step DIVA rtRTPCR which could be performed simultaneously in one reaction tube, employing systems for detecting simultaneously the vaccine and wild-type viruses.

Our approach is considered innovative as very few molecular DIVA assays have been described, including *Bluetongue* (6), *Bovine Herpesvirus, type 1* (7), *Classical Swine Fever virus*

Table 1: Details of the commercial flocks and amplification value (C_T) for TMEV by single- and duplex rtRTPCR DIVA

Flock no.	Disease onset (weeks of age)	Vaccination (weeks of age)	Signs	Monoplex DIVA		Duplex DIVA	
				General	1995/ vaccine	General	1995/ vaccine
29	14.0	9.0	+	24.7*	ND	24.2	ND
30	13.4	9.5	+	21.3	ND	20.9	ND
31	14.0	11.0	+	22.5	ND	22.5	ND
32	12.3	9.3	+	23.4	ND	22.9	ND
33	10.0	7.0	+	24.0	ND	23.2	ND
35	13.3	9.3	+	22.1	30.4, ND	18.6	31, ND
36	17.0	15.0	+	21.8	ND	22.2	ND
38	18.0	9.0	+	22.8	ND	22.0	ND
39	11.9	10.0	+	20.2	ND	20.2	ND
40	15.0	9.0	+	21.2	ND	21.5	ND
41	13.3	10.0	+	22.2	ND	23.5	ND
42	13.9	12.0	+	23.7	ND	23.9	ND
43	15.0	9.0	+	21.5	ND	21.2	ND
44	17.4	8.5	+	NT	NT	22.6	
46	16	9.0	+	NT	NT	20.5	
47	16.3	8.0	+	NT	NT	22.6	

* C_T value; ND – not detected; NT – not tested

(8), the *Infectious Laryngotracheitis virus* (ILTV) (9) and the quadruplex DIVA amplification of vaccine and wild-type Rift Valley Fever viruses (10).

The emergence of the cluster of clinically-affected turkey flocks in Israel during the summer-fall 2017 offered a unique opportunity to evaluate the diagnostic comparative performance of the duplex as opposed to the monoplex DIVA assay. The causative circumstances of the clinical events were not clear. However, as all flocks were vaccinated the identification of the underlying virus was crucial for epidemiological investigations. The present study presents this diagnostic tool and provides its relevant evaluation on commercial flocks.

MATERIALS AND METHODS

Turkey tissue samples

Brain tissues from various commercial turkeys affected with typical neurological symptoms were submitted for TMEV diagnosis. The flock age, vaccination status, etc. are detailed in Table 1. The brain tissues were excised and kept frozen for RNA purification and amplification.

RNA purification

RNA purification from turkey brain tissue was prepared using the Maxwell® 16 LEV simplyRNA Tissue Kit (Cat. No. AS1280) Promega Ltd., Madison, WI, U.S.A. according to the manufacturer' instructions. When compared to the previously reported purification method (4), using QIAmp® Viral RNA Mono Kit (QIAGEN Ltd., Valencia, CA, U.S.A., the presently used method of RNA purification was x10 more sensitive.

Monoplex and duplex rtRTPCR DIVA amplification

The rtRT-PCR amplification was performed as described previously (4), including the primers and probes for the General, a pan detection assay that detects both the vaccine and the wild-type viruses, and the 1995/vaccine strains (Table 2). However, the amplification mix component volumes were modified, such as to contain the probes and primers for the two systems. Briefly, the amplification mix at a volume of 20 µl contained 10 µl of qScript™ One-Step XLT RT-qPCR ToughMix Kit, ROX™ (Quanta BioSciences, Inc., Gaithersburg, MD, U.S.A.), 1 µl of each of the 4 primers (500 ηmol) and 2 probes (125 ηmol), and 2 µl of the RNA control, or the examined samples. In the monoplex assay, where only one primer pair, probe and RNA were included, the reaction volume was adjusted to the final volume with PCR-grade water. The cycling conditions were: 10 min. at 50^{0}C, 5 min. at 95^{0}C and 40 cycles of 10 sec. at 95^{0}C and 45 sec. at 60^{0}C. Assays were performed on a StepOne real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.).

RESULTS AND DISCUSSION

Comparative performances of monoplex and duplex DIVA rtRTPCR.

The sensitivity of detection and the amplification parameters of the monoplex and duplex DIVA rtRTPCR were analyzed simultaneously on the same sample of TMEV vaccine RNA. Seven ten-fold serial dilutions of the TMEV vaccine, starting

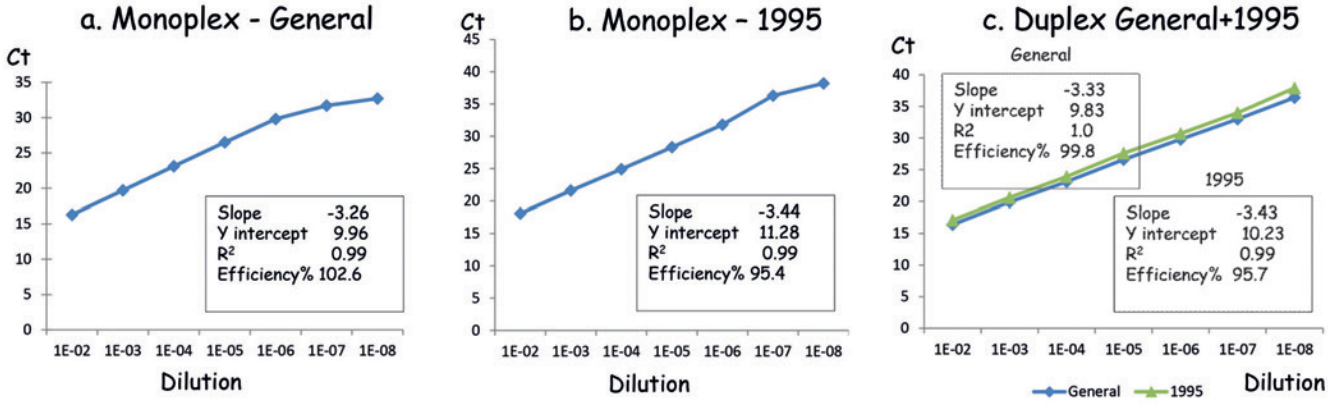

Figure: Amplification parameters of the single-plex and duplex TMEV DIVA rtRT-PCR on 7 ten-fold serial dilutions of the TMEV vaccine, starting from the RNA dilution of 10^{-2}, representing one vaccine dose, and up to the 10^{-8} dilution. A. The linear amplification curves for both DIVA systems, single-plex for the General (a), 1995/vaccine (b) or both as a duplex (c) are shown. The amplification parameters are detailed in Table 3.

Table 2: Primers and probes for the TMEV DIVA rtRTPCR

Strain	Name	Sequence
"General"	Forward	CGA GGA CAG TTG GTG TGG AA
	Reverse	CAG GGC TCT GAT CTG CAT GAT
	Probe	CAL Fluor Gold 540-CAC AGA ACA CGA TCA ACC TGG GCA GA-BHQ-1
"1995/vaccine"	Forward	ATG GGG TTC TGT AAG ATG TAA ATA ACT G
	Reverse	CCG GCC TGA CTC TCA AGT CC
	Probe	FAM-CAT AGA TGG AAT GTA GTG TTA GGC G-BHQ-1

from the 10^{-2} dilution were examined. The dilution starting point was an undiluted volume of RNA isolated from one dose of the original vaccine (EID_{50} – $10^{3.16}$/dose). The amplification parameters of the monoplex DIVA rtRTPCR using the General (Figure 1a) and the 1995/vaccine systems (Figure 1b), as well as the duplex rtRTPCR DIVA (Figure 1c) are detailed in Table 3. The parameters of both DIVA configurations were similar, therefore, the duplex assay could be further evaluated for diagnostic purposes.

Table 3: The amplification parameters of the single-plex and duplex DIVA systems

	Monoplex DIVA		Duplex DIVA	
	General	1995/vaccine	General	1995/vaccine
Slope	-3.26	-3.436	-3.328	-3.43
Y Intercept	9.961	11.279	9.825	10.226
R^2	0.999	0.996	1.0	0.999
Efficiency %	102.638	95.431	99.747	95.676

Evaluation of the monoplex and duplex DIVA rtRTPCR on the diagnosis of commercial turkey flocks.

The two DIVA configurations were evaluated for use in real clinical situations. The cluster of clinical cases that occurred during the summer-fall of the year 2017 offered an opportunity to compare the performance of the monoplex and duplex DIVA assays on the same clinical samples in both assays, for comparison. Table 1 shows the amplification values of 17 turkey commercial flocks. The samples were analyzed for diagnosis upon their arrival, each at a specific time, and then re-verified simultaneously, as a group, for both DIVA configurations to eliminate assay variability. As shown in Table 1, the amplifications were similar for all flocks by the two DIVA configurations, as mono- and as duplex for both the General and the 1995/vaccine TMEVs.

In conclusion, the rtRTPCR DIVA assay was now upgraded to a multiplex assay and showed to perform similarly to the monoplex configuration, both experimentally on the TMEV vaccine virus and both during the diagnosis of the 2017 TMEV outbreak. Although all flocks were vaccinated

with the TMEV live vaccine, the vaccine virus was not detected by the DIVA amplification. That feature might reflect the vaccine virus presence in very small quantities following vaccination, as demonstrated in our recent study (11). Only by using the nested rtRT-PCR could the TMEV vaccine virus be detected after commercial vaccination for a period of about two weeks post-vaccination (11). The TMEV DIVA systems, and the multiplex configuration, in particular, comprise not only a significant diagnostic advantage for its ability to identify one or more viruses in a concurrent infection, but also may stimulate future development of additional DIVA systems for other poultry viruses.

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