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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

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Abstract

The avian flavivirus Turkey Meningoencephalitis Virus (ITV) causes a neuroparalytic disease of commercial turkeys, expressed in paresis, incoordination, dropping wings and mortality that is controlled by vaccination. The newly developed ITV real time RT-PCR Differentiating Infected from Vaccinated Animals assay, was further developed and transformed into a one-step duplex assay to distinguish between wild-type ITV strains and vaccine virus and to help identify in one amplification the virus type involved in the recent emergence of ITV in the summer-fall of 2017. The performance of the newly developed duplex DIVA assay was equal to that of the two monoplex assays in detecting clinical cases evaluated on the recent outbreak that affected most of the commercial turkey flocks of the age of ITV clinical affection. Using the ITV 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 ITV-DIVA assay both as single-and duplex DIVA real-time PCR. In conclusion, a powerfully distinctive, sustainable and sensitive one-step duplex assay was put in action on the 2017 ITV outbreak.

Keywords: Avian Flaviviruses; DIVA; Duplex; Monoplex; Real-Time PCR; Turkey Meningoencephalitis Virus

Introduction

The Turkey Meningoencephalitis Virus (ITV), causes a neuroparalytic disease in adult turkeys leading to paresis, incoordination, dropping wings and mortality, due to inability to reach food and water. Vaccination of adult turkeys with a live-attenuated vaccine virus is practiced [1,2], but occasionally typical clinical signs are noticed, causing ambiguousness in diagnosis. Whether imperfect vaccination or poor vaccine efficacy might be the cause, a differential diagnosis of the causative virus might complement the diagnosis of affected commercial turkey flocks. ITV is an enveloped flavivirus, containing a 11-kb single-stranded positive-sense RNA genome, encoding for 3 structural (capsid (C), Pre-Membrane (prM) and Envelope (E), and 7 Non-Structural (NS) genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The initial phylogenetic classification of TMEV associated it with the mosquito-borne cluster, clade XI and to the Ntaya flavivirus antigenic complex VI [3]. TMEV has been detected in mosquitoes (Diptera: Culicidae), and Culicoides (Diptera: Ceratopogonidae) species and its survival in *Culex pipiens* and *Phlebotomus papatasi* (Diptera: Phlebotomidae) was demonstrated [4]. The whole genome sequencing of 5 ITV strains [5,6]. Facilitated the recent development of the ITV molecular diagnosis assay aimed to Differentiate Infected from Vaccinated Animals (DIVA). The Bagaza virus (BAGV) and TMEV isolates had 92-96% genomic and 99.7% amino acid homologies. A new look at the flavivirus taxonomy became evident, confirming that BAGV and TMEV are similar viruses, and may represent the same virus under different names. As ITV and Bagaza virus (BAGV) were shown as the same virus species, the present assay may also be applicable to

flaviviruses isolated under the denomination "Bagaza virus [5].

The initial TMEV molecular diagnosis was developed based on the simultaneous and independent elucidation of the first TMEV NS5 sequences [7]. covering different NS5 gene locations [8]. Since the env gene encodes for externally-exposed and selection-prone immunodominant proteins, the diagnostic PCR was based on the relatively conserved non-structural replication flavivirus NS5 gene [8]. To provide a detection assay for isolates in which possible genomic changes might have occurred in the env gene, a dual-gene end-point amplification RT-PCR, based on the NS5 and the TMEV envelope gene was developed [9] Development of ITV DIVA assays, as a conventional and a real-time RT-PCR (rtRTPCR) reflects a progress, replacing the two distinct amplifications, needed in order to detect differentially the ITV vaccine and field viruses [9,10].

We now 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. The DIVA development was motivated by the need to differentiate between affected birds as a result of vaccination, or as a result of infection with a TMEV field strain, and relied on whole genomic sequences of the TMEV vaccine and field viruses [5]. DIVA assays were initially developed to identify differentially antibodies to avian influenza viruses and vaccines [11]. Our approach is innovative as very few molecular DIVA assays were described, including Bluetongue [12], bovine herpesvirus, type 1 [13], Classical Swine Fever virus [5], the Infectious Laryngotracheitis virus (ILTV) [14] and the quadruplex DIVA amplification of vaccine and wild-type Rift Valley Fever viruses [15].

The emergence of the cluster of clinically-affected turkey flocks 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 are not clear. However, as all flocks were vaccinated the identification of the underlying virus is crucial for epidemiological investigations. The present study offers the diagnostic tool and provides its relevant evaluation on commercial flocks.

Methods

Organ Samples

Brain tissues from commercial turkeys affected with typical neurological symptoms were submitted for ITV diagnosis. A pool of tissues from different individuals in the same flock was analysed, and denoted flock. The flock age, vaccination status, etc. are detailed in (Table 1).

Flock no.	Disease onset (weeks)	Vaccination (weeks)	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							

Table 1: Details of the commercial flocks and amplification value (CT) for ITV by mono- and duplex rRT-PCR DIVA

The brain tissues were excised and kept frozen until 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 [10] using QIAmp® Viral RNA Mono Kit (QIAGEN Ltd., Valencia, CA, U.S.A., that method was x10 more sensitive.

Primers, Probes and Amplification

Primers and probes for the DIVA assay were designed from the TMEV NS5 gene of the 5 strains, for which the whole genome was elucidated (Table 2) [16].

Strain specificity	Name	Sequence
"General" – NS5 gene (*) (+)	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" NS5 gene (+)	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

Table 2: Primers and probes for the TMEV NS5 and env dual-gene and for the DIVA real-time amplification.

The "old isolate clade" included the vaccine virus strain, isolated in the year 1975 (KC734549) and the strain isolated in the year 1995 (KC734550), denoted "1995/vaccine". (Table 2) describes the primers and probes designed for the dual-gene and DIVA assays. The "general" rtRT-PCR and DIVA amplicon lengths were 246 bp and 240 bp, respectively. All amplifications were performed in duplicates. The rtRT-PCR amplification was performed as described previously [10], including the primers and probes for the General and the 1995/vaccine strains. 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 20µl contained 10µl of qScript TM One-Step XLT RT-qPCR Tough Mix Kit, ROXTM (Quanta Bio Sciences, Inc. Gaithersburg, MD, U.S.A.), 1µl of each of the 4 primers (500 nmol) and 2 probes (125 nmol), and 2µl 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 with water. The cycling conditions were: 10 min. at 500°C, 5 min at 950°C and 40 cycles of 10 sec. at 950°C and 45 sec at 600C. Assays were performed on a StepOne real-time PCR system (Applied Biosystems, Foster City, CA, U.S.A.).

Results

Establishment of The TMEV Duplex DIVA Amplification

The performance of the monoplex and duplex DIVA amplification was analysed using RNA purified from the commercial vaccine vial. Seven ten-fold serial dilutions of the ITV vaccine, starting 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 (103.16EID50/dose, 0.5 ml/bird). The sensitivity of detection and the amplification parameters of the monoplex and duplex DIVA rtRTPCR were analysed simultaneously on the same sample of ITV vaccine RNA which was diluted into seven ten-fold serial dilutions. 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) were mentioned on each graph. The parameters of both DIVA configurations were similar, therefore, the duplex assay could be further evaluated for diagnostic purposes.

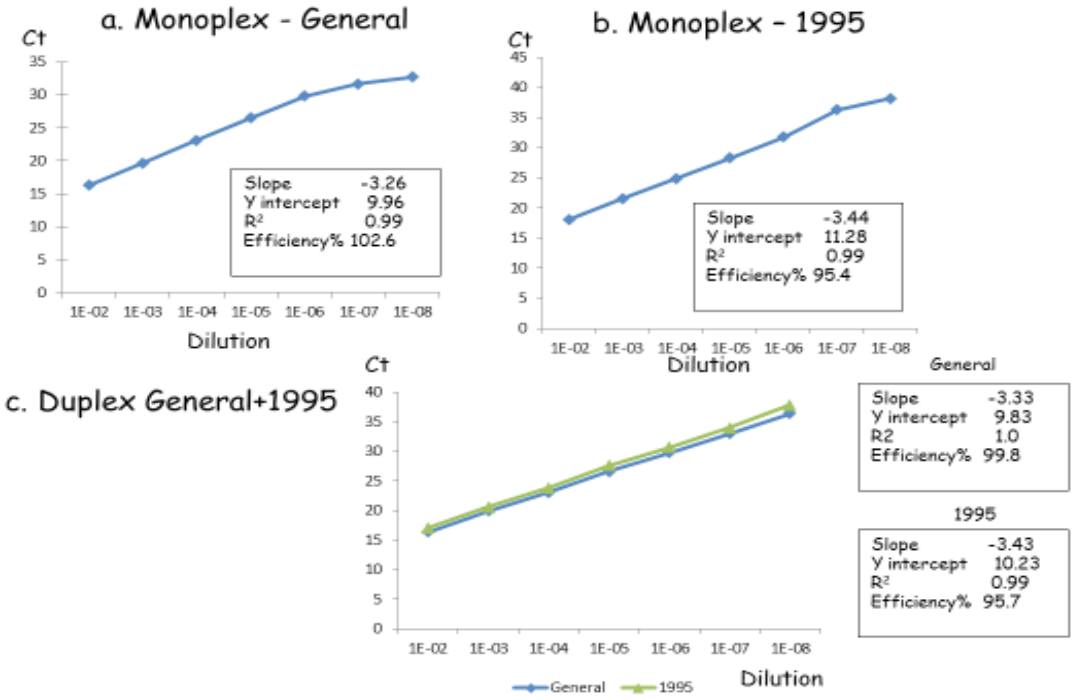

Figure 1: Amplification parameters of the monoplex and duplex ITV DIVA rtRT-PCR on 7 ten-fold serial dilutions of the ITV vaccine, starting from the RNA dilution of 10-2, representing one vaccine dose, and up to the 10-8 dilution. A. The linear amplification curves for both DIVA systems, monoplex for the General (a), 1995/vaccine (b), or both as a duplex (c) are shown. The amplification parameters are detailed in each section.

Application of The Duplex DIVA Amplification to Clinical Cases

The cluster of clinical cases that occurred during the fall of the year 2017 offered an opportunity to compare the performance of the monoplex and duplex DIVA assays on the same clinical samples. (Table 1) shows the amplification values of 17 turkey commercial flocks. The samples were verified upon their arrival for diagnosis, 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 ITVs.

Discussion

The rtRTPCR DIVA assay was now upgraded to a duplex assay and its performance was verified on ITV vaccine and field viruses from actual clinical cases in commercial flocks. The new assay performed similarly to the monoplex configuration, both experimentally, on the ITV vaccine virus, and both during the diagnosis of the 2017 ITV outbreak. Although all flocks were vaccinated with the ITV 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 [17]. Only by the nested rtRT-PCR the ITV vaccine virus could be detected after commercial vaccination for a period of about two weeks post-vaccination. Finally, the ITV DIVA systems, and the duplex configuration, in particular, comprise not only a significant diagnostic asset for its ability to identify one or more viruses in a concurrent infection, but also inspire future development of additional DIVA systems of other poultry viruses.

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