Journal of Virological Methods 185 (2012) 239–243

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

# Development of a reliable dual-gene amplification RT-PCR assay for the detection of Turkey Meningoencephalitis virus in Turkey brain tissues

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*Article history:*
Received 21 February 2012
Received in revised form 30 May 2012
Accepted 6 June 2012
Available online 13 June 2012

*Keywords:*
Turkey Meningoencephalitis virus (TMEV)
Flavivirus
NS5 gene
Envelope gene
Turkeys
Dual-gene amplification RT-PCR

A B S T R A C T

The Turkey Meningoencephalitis virus (TMEV) causes neuroparalytic signs, paresis, in-coordination, morbidity and mortality in turkeys. In parallel to the increased worldwide scientific interest in veterinary avian flaviviruses, including the Bagaza, Tembusu and Tembusu-related BYD virus, TMEV-caused disease also reemergence in commercial turkeys during late summer of 2010. While initially TMEV was detected by NS5-gene RT-PCR, subsequently, the *env*-gene RT-PCR was employed. As lately several inconsistencies were observed between the clinical, serological and molecular detection of the TMEV *env* gene, this study evaluated whether genetic changes occurred in the recently isolated viruses, and sought to optimize and improve the direct TMEV amplification from brain tissues of affected turkeys.

The main findings indicated that no changes occurred during the years in the TMEV genome, but the PCR detection sensitivities of the *env* and NS5 genes differed. The RT-PCR and RNA purification were optimized for direct amplification from brain tissues without pre-replication of clinical samples in tissue cultures or in embryonated eggs. The amplification sensitivity of the NS5-gene was 10–100 times more than the *env*-gene when separate. The new dual-gene amplification RT-PCR was similar to that of the NS5 gene, therefore the assay can be considered as a reliable diagnostic assay. Cases where one of the two amplicons would be RT-PCR negative would alert and warn on the virus identity, and possible genetic changes. In addition, the biochemical environment of the dual-gene amplification reaction seemed to contribute in deleting non-specific byproducts that occasionally appeared in the singular RT-PCR assays on RNA purified from brain tissues.



## 1. Introduction

The Turkey Meningoencephalitis virus (TMEV), denoted as ITV in the most recent International Committee on Taxonomy of Viruses (ICTV) report (9th) (King et al., 2012), attracted a renewed interest due to the disease reemergence in commercial turkeys in Israel during late summer of 2010. TMEV causes a neuroparalytic disease in turkeys leading to paresis, in-coordination, drooping wings and 15–30% mortality that can reach up to 80% of the flock. The virus was first described by Komarov and Kalmar (1960), identified as flavivirus by Portefield (1961) and classified phylogenetically to the mosquito-borne cluster, clade XI (Kuno et al., 1998) and Ntaya flavivirus antigenic complex VI (Calisher et al., 1989). Simultaneously and independently the TMEV NS5 genetic sequence were revealed (Davidson et al., 1998; Kuno et al., 1998), covering different NS5 gene locations (Davidson and Weisman, 2000). On the basis of the 3′ NS5 gene fragment (Acc. No. AF098456) Davidson et al. (1998) developed a specific RT-PCR assay to detect the virus in brain tissues, whose efficacy was demonstrated for the diagnosis of commercial flocks (Davidson et al., 2000). Further, Braverman et al. (2003) demonstrated the survival of TMEV in mosquito species in Israel, as well as the viability of the virus in experimental infection of the mosquitoes.

South Africa is the only country that TMEV was documented, except of Israel (Barnard et al., 1980). However, coincidently, several TMEV similar flaviriruses belonging to the Ntaya subgroup, have re-emerged in Spain, China, India and Malaysia; the Bagaza virus (BAGV) outbreak in wild partridges and pheasants in Spain (Aguero et al., 2011), the Tembusu virus (TMUV) (Cao et al., 2011; Yan et al., 2011a) and the Tembusu-related BYD virus (Su et al., 2011) outbreaks in Chinese ducks and the Sitiawan virus (SV) in Malaysian chickens (Kono et al., 2000). In addition, during a retrospective investigation of human encephalitis cases that occurred

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http://dx.doi.org/10.1016/j.jviromet.2012.06.001

during the year 1996 in the Kerala state in India, Bagaza virus was detected in mosquito pools and specific antibodies were identified in human sera (Bondre et al., 2009), supporting the previous foresight that implicated the Bagaza virus as one of the emerging and re-emerging human pathogens that causes febrile illness in humans (Woolhouse et al., 2006).

Recently the whole genomes sequencing of the Bagaza and Tembusu viruses were completed (Kuno and Chang, 2007; Tang et al., 2011a) and extensive phylogenetic characterization of the *Flavivirus* genus has been described (Kuno et al., 1998; Cook et al., 2011), showing the closest relatedness of TMEV to the Bagaza and slightly less to the Tembusu virus. The concurrent emergence of these viruses and of TMEV, which are closely related phylogenetically, is rather enigmatic, but could reflect either an increased awareness, changes in the entomological population of the insect vectors, or other unknown factors. Also, a common viral ancestor leading to the evolvement of very related and almost identical viruses, which evolved in various parts of the world and in different avian hosts, might be considered. According to Kuno et al. (1998) a similarity of >84% in the flavivirus gene sequences serves as a criterion for species delineation, in conjunction with virus neutralization activity, and can define virus similarity. Accordingly, the Blast analysis of the three available TMEV sequences (AF098456 and AF372415 of the TMEV NS5 gene and AF372415 of the TMEV *env* gene) show a sequence homology of 93%, 94% and 95%, respectively, to the Bagaza virus.

The present study describes the development of a dual-gene amplification RT-PCR for TMEV detection based on the NS5 (Davidson et al., 1998) and the TMEV envelope gene (Gaunt et al., 2001). Compared to the initially described diagnostic RT-PCR (Davidson et al., 2000), the new assay is an updated and reliable option for molecular diagnosis of TMEV. The TMEV identification based on two amplicons provides a validated detection, even if a possible genomic change might have occurred in the *env* gene, which is more variable compared to the conserved flavivirus NS5 gene that is active in replication. As the *env* gene encode for externally exposed immunodominant proteins, it is more prone to genetic changes due to selective pressures, as indicated by sequencing several isolates (data not shown).

## 2. Materials and methods

### 2.1. Viruses

The TMEV vaccine virus was the commercial TMEV vaccine strain was based on seed virus JQ4E4 described by Ianconescu et al. (1975). In addition to the commercial vaccine virus (A), several TMEV isolates served for experimental infections to obtain brain tissues to develop the diagnostic dual-gene amplification RT-PCR, as follows:

(B) Isolate 618 isolated in 1995 from a 10 weeks-old turkey
(C) Isolate 107458 isolated in 2010 from a 18 weeks-old turkey
(D) Isolate 106819 isolated in 2010 from a 14 weeks-old turkey
(E) Isolate 105520 isolated in 2010 from a 10 weeks-old turkey

Isolates 618 (B) and 107458 (C) are shown in Figs. 2 and 6 (see Sections 3 and 4), while the others were analysed but not shown.

### 2.2. Experimental TMEV infection

Apparently healthy commercial turkeys were grown in isolation from hatch until 9 weeks-old in separate groups and their sera was tested for TMEV antibodies by haemagglutination inhibition before inoculation. Each group was inoculated intra-cerebrally (i.c.) with 0.1–0.2 ml of TMEV inoculum using a 22G/1.5 in. needle. The inoculum, consisting of the TMEV isolates or the vaccine virus was 0.2 ml of 10% (w/v) mouse brain homogenate in PBS. The suspension was centrifuged at 1500 × *g* for 20 min followed by 10,000 × *g* for 20 min in a cooled centrifuge.

The mice passage step is an intermediate step of virus replication served to amplify the virus load and subsequent virulence, resembling the original disease. The mice inoculation step was performed as follows: the suspension, consisted of 10% (w/v) turkey brain homogenate in PBS, was injected into 1–5-day-old suckling mice. Injection is performed intra-cerebral (i.c.) at a 0.03 ml volume using a 0.3 ml insulin syringe. The mice are inspected daily; freshly dead and sick mice after euthanization were kept frozen. After experimental inoculation the turkeys showed clinical signs, including opistotomas, imbalance, morbidity and mortality commencing on day 3 p.i. until 10 days p.i. Brain tissues were kept frozen until RNA purification and amplification.

### 2.3. Turkey tissue samples

Brain tissues from various commercial turkeys at 2–3 months of age that were affected with typical neurological symptoms that were evident in Israel between the years 2008 and 2010 were submitted for TMEV diagnosis. The tissues were excised and kept frozen until RNA purification and amplification.

### 2.4. RNA purification

To optimize the RNA purification from turkey brain tissue for was purified by 3 purification methods according to the manufacturer's instructions:

A. SV Total RNA Isolation system (Promega, Madison, WI, USA).
B. RNA extraction from 10% (w/v) brain tissue homogenate in PBS using the QIAamp® Viral RNA Mini kit (Qiagene, Ltd., Valencia, CA. USA).
C. Automated RNA purification using the Maxwell 16 instrument using the 16 LEV simplyRNA Tissue kit (Promega, Madison, WI, USA).

To assess the most efficient method for RT-PCR amplification from the brain tissues for use in TMEV-RT-PCR, RNA was purified from the brain tissue of experimentally infected turkeys with the commercial vaccine virus by methods A and B, in parallel. RNA was purified from fragments of the same brain tissue and the specific activity (SA) was evaluated as follows: (a) the RNA concentration (ng/μl) was measured by the ND-1000 Nanodrop Spectrophotometer, (b) calculated per 1 mg of the original brain tissue that served for the RNA purification by each method, and (c) divided by reciprocal decimal factor of dilution at the NS5 RT-PCR endpoint. The SA values of methods A and B were 34.6 and 0.084, respectively, demonstrating a 400 higher SA in benefit for method A. Subsequently, automated RNA purification using the Maxwell 16 instrument was introduced using the 16 LEV simplyRNA Tissue kit (Promega, Madison, WI, USA) having a 25 fold higher SA compared to method B. Method C was chosen for all subsequent work, in view of the automation needed for application in diagnosis.

### 2.5. TMEV-NS5 gene amplification

RT-PCR was performed with Thermo Scientific Verso 1-step RT-PCR ReddyMix kit (ABgene Ltd., Surrey, UK) according to the manufacturer's instructions. First, 2.5 μl of RNA and 2.5 μl of $H_2O$ were heated to 94 °C for 2 min and then cooled and added to the RT-PCR mixture in final reaction volume of 25 μl containing 0.4 pmol of each primer. The cycling program included 15 min at 50 °C,