Trials in Vaccinology 3 (2014) 127–133

Contents lists available at ScienceDirect

Trials in Vaccinology

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

Virological confirmation of suspected dengue in a Phase 2 Latin American vaccine trial: Implications for vaccine efficacy evaluation

Mark Boaz [a,*], Helene Janosczyk [a], Seema Garg [a], Stacy Yost [a], Stephen W. Hildreth [a], Gustavo Dayan [b], Linda Starr-Spires [a]

[a] Global Clinical Immunology, Sanofi Pasteur, Discovery Drive, Swiftwater, PA, USA
[b] Clinical, Sanofi Pasteur, Discovery Drive, Swiftwater, PA, USA

ARTICLE INFO

Article history:
Received 4 June 2014
Revised 2 July 2014
Accepted 3 July 2014

Keywords:
Dengue
Vaccine
Virus
Virological confirmation
Laboratory algorithm
PCR

ABSTRACT

The CYD tetravalent dengue vaccine candidate is being evaluated for protective efficacy against symptomatic dengue in Phase 3 efficacy trials. The laboratory test algorithm to confirm dengue cases was evaluated prior to Phase 3 trials. During a Phase 2 trial in Latin America a dengue epidemic occurred in the study countries. A total of 72 suspected dengue cases were reported and assessed: virological confirmation comprised qRT-PCR methods and a commercial ELISA kit for NS1 protein (Bio-Rad). The qRT-PCR included a screening assay targeting a conserved dengue region of the 3′-UTR (dengue screen assay) followed by 4 individual serotype assays targeting the conserved dengue NS5 genomic region (WT dengue qRT-PCR assays). The NS1 and WT dengue qRT-PCR were endpoint assays for protocol virological confirmation (PVC). Of the 72 suspected cases, 14 were PVC. However, a unique pattern of dengue qRT-PCR results were observed in 5 suspected cases from Honduras: the dengue screen qRT-PCR assay was positive but WT dengue qRT-PCR and NS1 Ag ELISA were negative. To investigate these observations, additional molecular methods were applied: a SYBR® Green-based RT-PCR assay, sequencing assays directed at the genome regions covered by the WT dengue qRT-PCR, and a modified commercial dengue RT-PCR test (Simplexa™ Dengue, Focus Diagnostics). The exploratory data confirmed these additional cases as dengue and indicated the serotype 2 WT dengue qRT-PCR assay was unable to detect a circulating Latin American strain (DENV-2/NI/BID-V608/2006) due to a sequence variation in the isolate. The Simplexa Dengue RT-PCR test was able to detect and serotype dengue. Based on these findings an updated molecular test algorithm for the virological confirmation of dengue cases was developed and implemented in the Phase 3 efficacy trials.

Introduction

The dengue virus is a member of the Flavivirus (FV) genus, which also includes the yellow fever (YF) virus [1]. Dengue disease is caused by any of four dengue virus serotypes (serotypes 1–4), and is transmitted primarily by the *Aedes aegypti* mosquito [2]. Most dengue virus infections are asymptomatic, but clinical manifestations may range from mild febrile illness to severe and potentially fatal disease [2].

Dengue disease is endemic in the Caribbean, Central and South America, and Mexico, where the number of cases has increased dramatically in recent decades [3]. In 2011, nearly 1.1 million cases of dengue disease, including over 19,000 cases of severe dengue and more than 700 dengue-related deaths, were reported in Latin America [4].

No specific treatment exists for dengue disease, and preventive measures, which include mosquito control and personal protection from bites, have limited effectiveness. A recombinant, live-attenuated, tetravalent dengue vaccine (CYD-TDV; Sanofi Pasteur, Lyon, France) is under development containing four recombinant viruses (CYD-1–4) [5,6]. The CYD-TDV is currently in large scale Phase 3 efficacy trials in SE Asia and Latin America for prevention of symptomatic dengue. The evaluation of suspected dengue cases and diagnostic algorithms was assessed prior to Phase 3 in studies performed in endemic areas, as part of ensuring a robust standardized case definition for application in the Phase 3 efficacy studies.

* Corresponding author. Tel.: +1 570 957 2374; fax: +1 570 957 5644.
E-mail addresses: mark.boaz@sanofipasteur.com (M. Boaz), helene.janosczyk@sanofipasteur.com (H. Janosczyk), seema.garg@sanofipasteur.com (S. Garg), stacy.yost@sanofipasteur.com (S. Yost), steve.hildreth@sanofipasteur.com (S.W. Hildreth), Gustavo.dayan@sanofipasteur.com (G. Dayan), linda.starr-spires@sanofipasteur.com (L. Starr-Spires).

http://dx.doi.org/10.1016/j.trivac.2014.07.002
1879-4378/

Virological confirmation is performed with a two-step quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) algorithm consisting of an initial dengue screen qRT-PCR with subsequent serotyping by four serotype-specific wild-type (WT) dengue qRT-PCR assays, and/or with antigenic detection using NS1 Ag ELISA. In one such preparatory study, conducted primarily to evaluate the safety and immunogenicity of CYD-TDV in 9 to 16-year-olds in Latin America [7], unique observations were made with the protocol diagnostic algorithm versus previous observations from studies in SE Asia. The objective of this paper is to describe these observations in the Latin American Phase 2 trial, the subsequent tests performed to further characterize the circulating dengue cases, and subsequent adaptation of the diagnostic algorithm used for virological confirmation of dengue cases in clinical trials.

Materials and methods

Study design and sample collection

This study was a randomized, observer-blind (first and second injections), single-blind (third injection), controlled trial conducted in Colombia, Honduras, Mexico, and Puerto Rico (National Clinical Trials Identifier: NCT00993447) [7]. Healthy volunteers aged 9–16 years were randomized 2:1 to receive three subcutaneous injections of CYD-TDV or a control (placebo [0.9% NaCl] as the first and second subcutaneous injections and a tetanus/diphtheria/acellular pertussis [Tdap] vaccine as the third intramuscular injection). Injections were administered at 0, 6, and 12 months.

The study was conducted in accordance with the Declaration of Helsinki (Edinburgh, October 2000), Good Clinical Practice, International Conference on Harmonization guidelines and national and local ethical requirements. Written informed consent was obtained from participants and/or a parent or legal guardian. Symptomatic dengue cases were detected using surveillance of febrile episodes defined in alignment with the WHO case definition (e.g., temperature $\geqslant$38 °C on at least 2 consecutive days with a suspicion of dengue disease) [7].

Standard tests for virological confirmation of dengue

The tests performed to virologically confirm suspected dengue cases were those applied across all CYD vaccine studies and included validated qRT-PCR assays, and a commercially available enzyme-linked immunosorbent assay (ELISA) against the dengue non-structural protein 1 (NS1) antigen (Platelia™, Biorad Laboratories, Marnes-La-Coquette, France). The qRT-PCR testing for all suspected dengue cases is performed centrally at the sponsor's Global Clinical Immunology laboratory in a GCLP-like environment, with laboratory personnel blinded to the study groups, and include a screening assay targeting a conserved dengue region of the 3′-UTR (Dengue screen assay; adapted from [8]), followed by four individual serotype assays that utilize specific primers and probes for each dengue serotype targeting conserved dengue NS5 genomic regions (WT dengue qRT-PCR assays). The NS1 and WT dengue qRT-PCR were endpoint assays for protocol virological confirmation (PVC) (Fig. 1).

Additional tests to characterize virological dengue

Additional testing conducted post hoc using molecular methods to further characterize the dengue cases included a SYBR® Green-based RT-PCR assay, sequencing assays directed at the dengue genomic regions covered by the dengue qRT-PCR assays, and a modified commercial dengue RT-PCR test (Simplexa™ Dengue, Focus Diagnostics, CA, US). These additional tests were performed by laboratory personnel blinded to the study groups, in samples from suspected dengue cases that had consented to tests for research purposes, and that fulfilled the following criteria: a dengue screen qRT-PCR positive result according to the protocol definition ($\geqslant$LLOQ), or that had a detectable result which could not be quantified (>LOD). As an internal control, one sample that was NS1 antigen positive with no RNA quantified by the Dengue screen qRT-PCR was included in testing. The RNA used in these experiments were from the extractions performed from subject sera for the standard protocol virological testing described above, except for the Simplexa Dengue testing, which used freshly extracted RNA from different aliquots of the same sera.

The SYBR Green-based RT-PCR assay was performed by substituting SYBR Green in place of the probes. This method, using the serotype specific primers from the WT dengue qRT-PCR assays, permits the SYBR Green dye to intercalate into any double-stranded DNA, emitting a fluorescent signal detectable by the thermalcycler.

To assess changes to the assay-specific genomic regions, sequencing of the NS5 regions was performed on an ABI 3130XL system (Life Technologies, CA, USA), using the amplicons produced by each WT dengue qRT-PCR assay. The results from the sequencing reactions were compared to 950 dengue 2 sequences from Latin America, the Caribbean and Asia retrieved from the National Center for Biotechnology Information (NCBI) database using a Basic Local Alignment Search Tool (BLAST). The commercial dengue RT-PCR test (Simplexa Dengue, Focus Diagnostics) was performed following an adapted version of the manufacturer's instructions.

A phylogenetic tree was conducted using FastTree software (Lawrence Berkeley National Lab, US) from a multiple sequence alignment performed with the Muscle algorithm [9]. The phylogenetic tree was customized using Dendroscope software (University of Tubingen, Germany). The NS5 coding sequence corresponding to nucleotides 7548–10,247 of the dengue 2 isolate: DENV-2/NI/BID-V608/2006 (accession number EU596483.1) was aligned for dengue serotype 2 isolates obtained in Latin America and the Caribbean.

Results

Protocol-defined virological test results

Of subjects with suspected dengue, 14 were positive by virological confirmation (PVC) according to protocol endpoint assays although 5 could not be serotyped by RT-PCR (Table 1, subjects #1–14 and 2, 7, 8, 13, 14, respectively). A further 5 samples had virological test results indicative of a recent Dengue infection but were positive in the Dengue screen qRT-PCR only and not other protocol virological testing (Table 1, subjects #15–19).

Exploratory analysis

Additional molecular methods were applied to serotype the remaining 5/14 PVC cases and to assess the 5 additional samples that were positive in the Dengue screen qRT-PCR only. In addition, 7/9 of the serotyped PVC cases were available for exploratory testing and were included to help assess these additional molecular methods (Fig. 2).

Seven of the 10 non-serotyped qRT-PCR positive cases were from Honduras (Table 1, subjects #13–19) suggesting that circulating Honduran strains exhibited sequence differences that prevented detection in the standard in-house serotyping assay (WT dengue qRT-PCR assay). To assess the potential impact of sequence changes in the probe region, we tested these samples with a SYBR