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Research paper

Heparin removal by ecteola-cellulose pre-treatment enables the use of plasma samples for accurate measurement of anti-Yellow fever virus neutralizing antibodies

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ABSTRACT

Technological innovations in vaccinology have recently contributed to bring about novel insights for the vaccine-induced immune response. While the current protocols that use peripheral blood samples may provide abundant data, a range of distinct components of whole blood samples are required and the different anticoagulant systems employed may impair some properties of the biological sample and interfere with functional assays. Although the interference of heparin in functional assays for viral neutralizing antibodies such as the functional plaque-reduction neutralization test (PRNT), considered the gold-standard method to assess and monitor the protective immunity induced by the Yellow fever virus (YFV) vaccine, has been well characterized, the development of pre-analytical treatments is still required for the establishment of optimized protocols. The present study intended to optimize and evaluate the performance of pre-analytical treatment of heparin-collected blood samples with ecteola-cellulose (ECT) to provide accurate measurement of anti-YFV neutralizing antibodies, by PRNT. The study was designed in three steps, including: I. Problem statement; II. Pre-analytical steps; III. Analytical steps. Data confirmed the interference of heparin on PRNT reactivity in a dose-responsive fashion. Distinct sets of conditions for ECT pre-treatment were tested to optimize the heparin removal. The optimized protocol was pre-validated to determine the effectiveness of heparin plasma:ECT treatment to restore the PRNT titers as compared to serum samples. The validation and comparative performance was carried out by using a large range of serum *vs* heparin plasma:ECT 1:2 paired samples obtained from unvaccinated and 17DD-YFV primary vaccinated subjects. Altogether, the findings support the use of heparin plasma:ECT samples for accurate measurement of anti-YFV neutralizing antibodies.
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1. Introduction

Technological innovations in vaccinology have recently contributed to bring about novel insights for understanding the broader and complex interactions involving the vaccine-induced immune response (Pulendran, 2009, 2014; Nakaya and Pulendran, 2012; Mooney et al., 2013). The biological systems framework associated with immunomics strategies have been employed to determine the immunological

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signatures and provided a detailed mapping and integrative analysis to predict the behavior of multiple biological events triggered upon vaccination (Querec et al., 2009; Nakaya and Pulendran, 2012, 2015; Mooney et al., 2013).

Human peripheral blood is a rich biological material that allows for the simultaneous investigation of several elements employing distinct platforms of microassays to analyze antibodies, soluble factors, proteins, metabolites, cell subpopulations, RNA and DNA (Nakaya and Pulendran, 2012, 2015). While the current protocols that use peripheral blood samples can generate abundant data, a range of distinct whole blood derivatives (serum, plasma and cellular components, microparticles, etc.) are required to assemble a multi-functional and integrated analysis of cellular and humoral immunological biomarkers. To reach this goal, the proper collection of whole blood samples without and with distinct anticoagulant agents may be necessary for proper parametric analysis data assessment.

In general, samples without anticoagulant pose a challenge for the availability of cellular components. Likewise, different anticoagulant systems (ethylenediaminetetraacetic acid – EDTA, heparin, citrate, oxalate and fluoride) can impair some properties of the biological sample and/or interfere with functional assays, stressing the importance of proper choice of method for blood collection (Tong et al., 2016; Wisgrill et al., 2016). Usually, heparin is the choice for assaying cellular immunity, because it is a natural anticoagulant found *in vivo*, allowing proper performance of functional tests since it does not affect the subsequent process of cell activation dependent on calcium (Engstad et al., 1997; Young and Bermes, 1999). However, heparin can interfere with functional assays, such as detecting antibodies specific for various molecular targets in biological samples. The interference of heparin in functional screening assays of viral neutralizing antibodies has been well characterized (Harrop et al., 2004). Several studies have shown that heparin binds to viral particles, changing the neutralization profile of assays (Chen et al., 1997; Guibinga et al., 2002; Harrop et al., 2004; Hughes et al., 2014; Lin et al., 2014). In fact, previous studies have shown that the presence of heparin in plasma samples leads to false-positive results because it increases the ability of a biological sample to block viral association and entry in functional assays to detect neutralizing antibody (Harrop et al., 2004).

Therefore, the ideal scenario for a wide and polyfunctional evaluation of the use of blood components would require parallel collection of multiple peripheral blood samples, in the absence and presence of different anticoagulant systems. This ideal scenario could be carried out in studies involving adult humans or large experimental models, in which the collection of substantial volumes of peripheral blood is not an ethical/operational limitation. However, studies in human infants, children or small animal models differ greatly from that ideal situation and usually restrict the blood collection possibilities. In such cases, it is crucial to prioritize the sample collection system. In order to solve these limitations, several studies have been developed to establish pre-analytical treatment protocols of biological samples for the simultaneous investigation of cellular and humoral components in functional immunomics applied in vaccinology. Several protocols feasible for removal of anticoagulants, such as heparin sodium, have been described previously, such as enzymatic elimination or heparin adsorption to ecteola-cellulose (ECT) slurry (Thompson and Counts, 1976; Cumming et al., 1986; van den Besselaar and Meeuwisse-Braun, 1993; Hendrikx et al., 1997; Sánchez-Fito and Oltra, 2015). The term ecteola resin refers to modified cellulose which contains active basic substituents introduced into the cellulose molecule by reaction with epichlorohydrin and triethanolamine. Although widely available and relatively standardized to completely remove different amounts of heparin (0.1 and 1.0 IU/mL), the protocols available for ECT treatment do not cover all of the heparin concentration ranges (≥20 IU/mL) that can be reached when collecting blood samples with vacuum collection systems that can yield differing final volumes (Thompson and Counts, 1976). These remaining gaps in the pre-analytical treatment protocols of biological samples still requires further investigation to establish optimized protocols. The present study intended to optimize and evaluate the performance of pre-analytical treatment of heparin plasma samples with ECT with emphasis on technological innovation applied in the studies of protective immunity triggered by 17DD-YFV vaccine. The accurate measurement of anti-YFV neutralizing antibodies, by the functional plaque-reduction neutralization test (PRNT) has been considered the reference method to measure the immune response to vaccination and monitor the protective immunity induced by the YFV vaccine (Niedrig et al., 1999; Simões et al., 2012). The results generated support the use of heparin plasma:ECT treatment to remove the heparin interference observed in the PRNT test, making the heparin plasma samples suitable for accurate measurement of anti-YFV neutralizing antibodies.

2. Materials and methods

2.1. Study population

This study was submitted to the Ethics Committee for studies with human subjects at Centro de Pesquisas René Rachou - FIOCRUZ-Minas (Plataforma Brasil # CAAE 47136115.0.0000.5091) and approved under the protocol # 1.249.195 from September, 29th, 2015. The experimental design and protocols were proposed by the Collaborative Group for Studies of Yellow Fever Vaccine. A total of 1190 samples were included in the present investigation, 236 from unvaccinated subjects and 954 from 17DD-YFV vaccinated subjects, from both genders, age ranging from twelve months to seventy years. Unvaccinated subjects comprised residents from Vitória, ES, Brazil, located in a region without Yellow

Fig. 1. Panoramic overview of the experimental design and standard operational procedures. The optimization of ecteola-cellulose treatment to enable the use of heparin plasma samples in anti-Yellow fever virus neutralization antibody test (PRNT) was carried out in three steps, including: I. Problem statement; II. Pre-analytical steps; III. Analytical Steps. I. Problem statement intended to verify the interference of anticoagulants on PRNT using paired serum, EDTA and heparin plasma samples (n = 273), collected from 17DD-YFV vaccinated subjects. PRNT titers were assayed on microplate test according to Simões et al. (2012). II. Pre-analytical steps consisted of two experimental batches referred as: (II.I) Base/acid ecteola-cellulose slurry activation. The reagents and the overall procedure for ecteola-cellulose slurry activation is provided in the figure; and (II.II) Interference of heparin on PRNT and set of conditions of ecteola-cellulose treatment. The impact of heparin on PRNT were tested using paired samples, including serum *vs* heparin plasma (n = 84), obtained from unvaccinated and 17DD-YFV primary vaccinated subjects. The impact of distinct heparin plasma concentrations (20, 40, 80, 160 and 320 USP units/mL) were also tested using paired samples, including: serum *vs* $_{\text{sequential}}$heparin concentration (n = 54), obtained from unvaccinated and 17DD-YFV primary vaccinated subjects. Three sets of conditions were assembled (heparin plasma:ECT 1:1; 1:2 and 1:4) to choose the best heparin plasma:ECT treatment proportion, testing paired serum *vs* heparin plasma:ECT samples (n = 32), obtained from 17DD-YFV primary vaccinated subjects. The summary of the overall procedure for ecteola-cellulose treatment is provided in this figure. III. Analytical steps comprised two approaches referred to as: (III.I) Pre-validation analysis & cross-check. The pre-validation intended to determine the effectiveness of heparin plasma:ECT treatment to restore the PRNT titers as compared to serum samples. Sets of paired samples were tested, including: serum, heparin plasma and heparin plasma:ECT 1:2 (n = 39), obtained from 17DD-YFV primary vaccinated subjects. The cross-check analysis intended to verify whether the ECT treatment would lead to sample dilution. Sets of paired samples were tested, including: serum, serum:ECT 1:2, serum:PBS 1:2, heparin plasma, heparin plasma:ECT 1:2 and heparin plasma:PBS 1:2 samples (n = 60), obtained from 17DD-YFV primary vaccinated subjects; and (III.II) Validation & comparative performance. The validation was carried out using a range of paired samples, including: serum *vs* heparin plasma *vs* heparin plasma:ECT 1:2 (n = 324), obtained from unvaccinated and 17DD-YFV primary vaccinated subjects. The comparative performance was carried out by using serum *vs* heparin plasma:ECT 1:2 paired samples (n = 324) collected from unvaccinated and 17DD-YFV primary vaccinated subjects. This approach intended to check the cut-off and calculate the performance indexes (sensitivity, specificity and *kappa* agreement). A total of 1190 samples were included in the study. Duplicates of 9520 PRNT tests were performed to support the use of heparin plasma:ECT samples for accurate measurement of anti-YFV neutralizing antibodies.

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