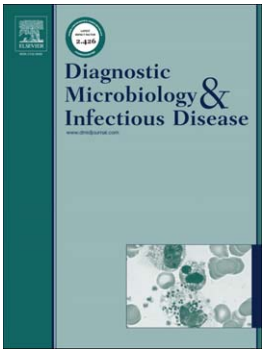

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PII: S0732-8893(16)30394-7
DOI: doi: 10.1016/j.diagmicrobio.2016.11.011
Reference: DMB 14242

To appear in: *Diagnostic Microbiology and Infectious Disease*

Received date: 5 May 2016
Revised date: 28 October 2016
Accepted date: 19 November 2016

Please cite this article as: Ramakrishnan Girija, Wright Marcia, Alam Masud, Naylor Caitlin, Kabir Mamun, Zerin Ayesha, Ferdous Tahsin, Pedersen Karl, Hennig Branwen J., Donowitz Jeffrey R., Wegmuller Rita, Haque Rashidul, Petri Jr William A., Herbein Joel, Gilchrist Carol A., Rapid Assessment of Tetanus Vaccine-induced Immunity in Bangladesh and The Gambia, *Diagnostic Microbiology and Infectious Disease* (2016), doi: 10.1016/j.diagmicrobio.2016.11.011

Rapid Assessment of Tetanus Vaccine-induced Immunity in Bangladesh and The Gambia

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Running Head: Rapid tetanus dipstick test for vaccine efficacy assessment

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ABSTRACT[1]

We have developed recombinant fragment C based rapid point of care dipstick devices to assess tetanus immunization status using plasma or whole blood. The devices demonstrated specificity of 0.90 and sensitivity of 0.90 (whole blood) / 0.94 (plasma) at field sites in Bangladesh and The Gambia when compared to a commercial ELISA with the immune cut-off titer set as ≥ 0.1 IU/mL.

Keywords: vaccine, tetanus, dipstick, lateral flow, fragment C

[1] **Abbreviations**
Global Vaccine Action Plan (GVAP); Diphtheria-tetanus-pertussis (DTP3); recombinant Fragment C (rFragC); false negatives (FN); true positives (TP); inter quartile range (IQR)

Childhood vaccination forestalls an estimated 2 to 3 million deaths annually, however preventable diseases like maternal and neonatal tetanus remain a health problem in many parts of the world (1). A recent analysis of childhood mortality placed the burden of neonatal tetanus at 49,000 in 2013, accounting for 1.7% of all neonatal deaths (2). Immunization campaigns have been pivotal in reducing the incidence of this disease (3). Women of reproductive age are routinely given tetanus vaccine as part of prenatal care services and during campaigns in high-risk areas, while children receive three doses of diphtheria-tetanus-pertussis (DTP) vaccine in the first year of life. Despite progress, the goal of the Global Vaccine Action Plan (GVAP) to achieve 90% DTP3 vaccination coverage worldwide by 2015 has remained unmet and monitoring of immunization at the country level has been recommended by the Strategic Advisory Group for GVAP (4). A validated test of immune status that is affordable and easy to deploy would assist in monitoring immunization programs and identifying populations that are susceptible due to lack of vaccination or due to poor vaccine efficacy (5).

Indirect ELISA is widely used in clinical settings to detect the presence of vaccine-induced anti-tetanus antibodies in either whole blood or plasma. We previously validated the use of recombinant Fragment C (rFragC) of the tetanus toxin as an alternative antigen to tetanus toxoid in ELISA (7). We have now developed dipstick tests using rFragC as a rapid and less expensive alternative to the available commercial ELISAs. The new dipstick devices use gold-conjugated rFragC to bind anti-tetanus antibodies in blood or plasma/serum.

These complexes are carried by lateral flow of the assay diluent up the dipstick and trapped by immobilized rFragC in the dipstick detection zone to generate a red-colored antigen-antibody-antigen sandwich signal when the plasma antibody titer is ≥ 0.1 IU/mL. For the plasma dipstick device, 20 μL of plasma is mixed into 580 μL of diluent in a tube before introduction of the dipstick to start the lateral flow and for the whole blood dipstick device, 40 μL of blood is spotted on the sample pad before placing the dipstick in 360 μL of diluent to start the flow. The results are discerned by eye as positive or negative by the visual detection of a band after 20 minutes, which may also be quantified using a lateral flow reader if required. WHO anti-tetanus IgG standards supplied by NIBSC, UK were used to calibrate the device so that a signal could be discerned by eye at ≥ 0.125 IU/mL. Whole blood and plasma samples commercially obtained in the USA (Bioreclamation IVT, New York, USA) were used to initially optimize device performance and assess reproducibility.

Whole blood and plasma samples obtained at field sites of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) and of the Medical Research Council Unit, The Gambia (MRCG) were used to validate these devices. In Bangladesh, children (n=104) in an urban slum in Dhaka who had been vaccinated with a pentavalent vaccine (EPI-Bangladesh combined vaccine: diphtheria, pertussis, tetanus, hepatitis B, *Haemophilus influenzae* type b) as part of a longitudinal icddr,b study of child health (8–10) were assessed at 53 and 104 weeks of age. The Gambian cohort comprised children (n=174) from villages in the Kiang West region who were aged five or

under and had received the pentavalent vaccine administered by MRCG at the Keneba rural field station in line with the Gambian EPI schedule (11). Collection of blood from a small number of adult males (n=20) belonging to the same Gambian rural population was included in our study protocol with the aim of obtaining tetanus-vaccine naïve samples for assessing specificity of the dipsticks. The studies were approved by the relevant authorities: the Ethical Review Committee at icddr,b or The Gambia Government/MRC Joint Ethics Committee. A reliance agreement was executed between the reviewing committees and the University of Virginia Institutional Review Board.

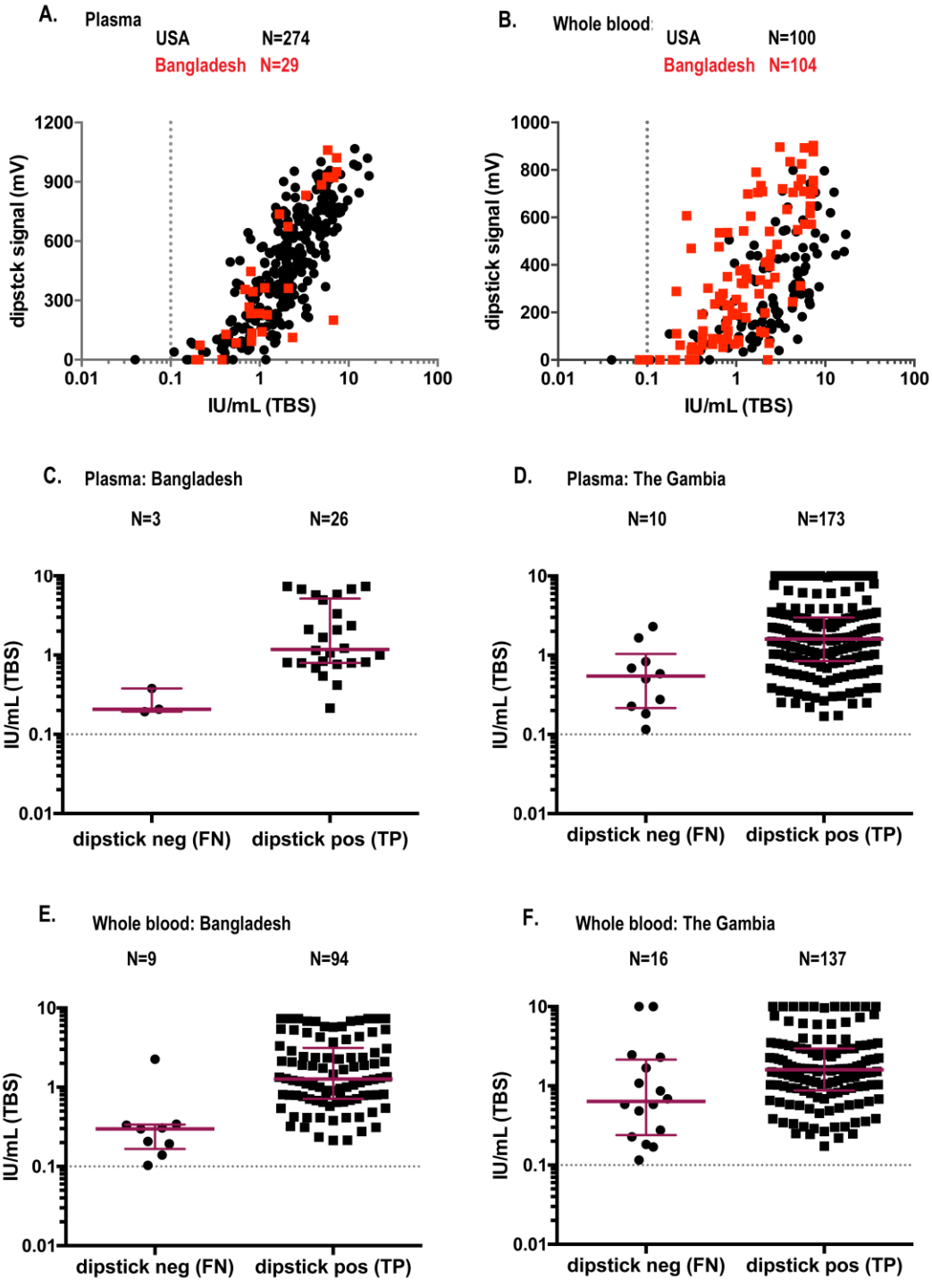
Plasma testing: Plasma samples were prepared by centrifugation of whole blood in EDTA tubes, and plasma was either assayed immediately or rapidly frozen at -80^{o}C until use. We first used samples from USA (n=274) and Bangladesh (n=29) to assess performance of the plasma device. The dipstick signal (quantified as peak height in mV using the ESEQuant reader (Qiagen Inc., USA) or evaluated by eye as positive/negative) was compared to the corresponding anti-tetanus titer in plasma determined in IU/mL using a standard toxoid-based ELISA (TBS, The Binding Site, Birmingham, UK). While titers as low as 0.01 IU/mL could be protective an ELISA-derived value >0.16 IU/mL was found to more reliably predict protection (13). WHO considers that a titer of 0.1-0.2 IU/mL correlates with protection in a ELISA (14). For our analysis, we considered 0.1 IU/mL to be the immune cutoff in the TBS ELISA and assigned a value of 0.01 IU/mL or 10 IU/mL to samples that fell below or above the range of the standard curve (0.01-7.0 IU/mL). The dipstick results showed good correlation of the signal quantified

as peak height with log-transformed anti-tetanus titer (Fig. 1A) and yielded a Spearman correlation coefficient (r_s) of 0.8 and 0.79 with samples from US and Bangladesh, respectively.

We then assessed the devices at both field sites using the visual read-out as an indication of immune status (Bangladesh n=29; The Gambia n=194). The TBS positive titers at the two sites had median values of 1.23 and 1.49 IU/mL respectively. Although only one Gambian child was non-immune by TBS assay, a total of thirteen of the TBS positive samples from both sites tested negative with the dipstick device. These false negatives (FN) had a median value of 0.2 IU/mL at the Bangladesh site (n=3) and 0.54 IU/mL at The Gambia site (n=10) (Fig. 1C, D). This apparent discrepancy in FN's may be due to differences in the operation of the test at the two sites; independent confirmation of weak test signals was routinely done at icddr,b but was not practical at MRCG.

Whole blood testing: The dipstick device performance with blood was initially assessed for correlation of signal strength with plasma anti-tetanus titers using USA samples (n=100) and with freshly collected samples at the Bangladesh site (n=104) (Fig. 1B). Whole-blood dipstick results were then evaluated by eye at the two field sites (Bangladeshi Children, n=104; Gambian Children, n=143; Gambian Adults, n=20). Nine adults and one child were non-immune by TBS ELISA. Possibly due to the operational differences at the two sites described above, FNs had median values of 0.3 IU/mL at the Bangladesh site (n=9) and 0.63 IU/mL at The Gambia site (n=16) (Fig. 1E, F)

Figure 1. Performance of tetanus dipstick devices. **(A,B)**. Correlation of plasma and blood device signals with TBS IU/mL. The dipstick signals with plasma (A) and blood (B) samples from a USA cohort (black dots) or a Bangladeshi cohort (red dots) were quantified in mV using a lateral flow reader and the signal peak heights (y-axis) were plotted against anti-tetanus ELISA titers (x-axis(log)) using Prism 6.0 software (GraphPad Software, San Diego, CA). The Spearman rank correlation was used in analysis of the data (A) Plasma: USA (r_s=0.8, P<0.0001, N= 274); Bangladesh (r_s =0.79, P<0.0001, N= 29); (B) Blood: USA (r_s =0.67, P<0.0001, N= 100); Bangladesh (r_s =0.75, P<0.0001, N= 104). **(C-F)**. Effectiveness of dipstick in discrimination of tetanus immune plasma (C,D) and blood (E,F) from cohorts in Bangladesh (C,E) and The Gambia (D,F). Anti-tetanus titers of dipstick negative (false negatives, FN) and positive samples (true positives, TP) are plotted and inter quartile range (IQR) shown. The dotted line indicates the cut-off for protection at 0.1 IU/mL. (C) Bangladesh plasma: FN (N=3, median=0.207 IU/mL, IQR=0.193-0.379 IU/mL); TP (N=26, median=1.18 IU/mL, IQR=0.8-5.17 IU/mL) (D) Gambian plasma: FN (N=10, median=0.54 IU/mL, IQR=0.22-1.03 IU/mL); TP (N=173, median=1.6 IU/mL, IQR=0.84-3.0 IU/mL) (E) Bangladesh blood: FN (N=9, median=0.3 IU/mL, IQR=0.17-0.34 IU/mL); TP (N=94, median=1.27 IU/mL, IQR=0.71-3.14 IU/mL) (F) Gambian blood: FN (N=16, median=0.63 IU/mL, IQR=0.24-2.14 IU/mL); TP (N=137, median=1.6 IU/mL, IQR=0.87-2.95 IU/mL).



We combined the results from visual readout at the two field sites for assessing the sensitivity and specificity of the dipsticks with whole blood and with plasma as substrates (Table 1).

Table 1. Operational characteristics and assay performance of dipstick devices compared to TBS ELISA results (Bangladeshi and Gambian populations, merged data)

Performance characteristics	Plasma	Whole blood
Sensitivity (95%CI)	0.94 (0.9-0.97) (n=212)	0.90 (0.87-0.94) (n=256)
Specificity (95% CI)	0.90 (0.59-1.00) (n=11)	0.91 (0.59-1.00) (n=11)
Positive Predictive Value	0.99	0.99
Negative Predictive Value	0.43	0.29

The dipstick devices had a sensitivity $\geq$0.9 and positive predictive value of 0.99 with both plasma and whole blood from >200 tetanus–immune individuals in our study cohorts. We were able to identify 11 non-immune individuals; samples from 10 of these tested negative with our dipstick devices, yielding a specificity of 0.9. The low negative predictive value (plasma = 0.43; blood = 0.29) reflects the number of FN in our testing as well as the small number of unvaccinated individuals in our study cohorts (TN) (15) which is a limitation of the study. Our results indicate that these tetanus dipstick devices are well-suited for deployment using either plasma or blood in vaccination outreach programs, allowing the

identification of non-immune or low titer individuals for whom booster shots are recommended (16). However, as is the case with using indirect ELISA as a diagnostic, individuals with anti-tetanus titers lower than 0.1 IU/mL are assessed by the dipstick devices to be non-immune although levels as low as 0.01 IU/mL are known to be protective. However, our experience has been that vaccinated individuals most commonly possess titers >0.1 IU/mL. Nevertheless, these tests hold potential for rapid and easy point of care assessment of immunity to tetanus particularly in community-based surveys, and would be effective even in resource-poor settings using a drop of blood obtained from a heel or finger prick.

Acknowledgments

We are grateful for the willing participation of the parents and children at the icddr,b and the MRC Keneba study sites. We also thank the field workers, nurses, laboratory assistants and data office staff at these sites for their assistance with this work.

Funding Information

This work was supported by the Bill and Melinda Gates Foundation (OPP #1066629) and by the NIH (5R01 AI043596-16). Funding to the MRC International Nutrition Group comes from the UK Medical Research Council (MRC) and the UK Department for International Development (DFID) under the MRC/DFID Concordant agreement (current grant MC-A760-5QX00).

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Highlights

- Point of care evaluation of vaccine-induced anti-tetanus antibodies
- No cold chain requirement
- Two dipsticks optimized either for testing serum or blood
- The devices were tested at two field sites: icddr,b, Bangladesh and MRC Keneba, The Gambia
- The dipsticks compared favorably with the results from a commercial ELISA