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Comparative evaluation of the antibody in lymphocyte supernatant (ALS) and enzyme-linked immunospot (ELISPOT) assays for measuring mucosal immune responses to *Shigella* antigens

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Abstract

Accurately assessing mucosal immune responses to candidate vaccines remains a technical challenge. ELISPOT is widely used as a surrogate of mucosal immune response by directly enumerating circulating antibody secreting cells (ASC), while antibody in lymphocyte supernatant (ALS) titers the total amount of antibody secreted by ASC *ex vivo* using ELISA. ALS is more practical than ELISPOT because the ASC supernatant is frozen for ELISA that can be conducted at any time, with any antigen, and in any laboratory. We compared IgA and IgG responses to serotype-specific *Shigella* LPS using ELISPOT and ALS in subjects following vaccination or infection with *Shigella*. ALS results correlated well with ELISPOT results, and the ALS method was both sensitive and specific for the detection of antibody responses against *Shigella* LPS. Based on these observations, the ALS assay is a practical and flexible alternative to ELISPOT for measuring mucosal IgA responses to *Shigella* LPS antigen.

Keywords

Shigella; mucosal immune response; ELISPOT; ALS

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1. Introduction

Shigellosis is an enteric infection of global public health and clinical importance, causing approximately 165 million cases of disease every year [1]. The global emergence of drug-resistant strains of *Shigella* emphasizes the need for accelerated development of vaccines to prevent disease in people living in developing countries and in military personnel and travelers to *Shigella* endemic areas [1–3]. While clinical trials of potential vaccine candidates for shigellosis are ongoing [4], an unresolved issue is how best to judge mucosal immune responses to vaccine antigens.

Direct methods for assessing mucosal immune responses to vaccine antigens have proven to be cumbersome for study subjects and laboratory staff.[5–7] Options for indirect measure of mucosal immune responses are generally more practical and these include the widely used enzyme-linked immunospot (ELISPOT) and the newer antibody in lymphocyte supernatant (ALS) assays.[8] Antibody secreting cells (ASC) stimulated at one mucosal site transit through the peripheral blood to other mucosal sites and reach peak levels in the peripheral blood approximately one week after stimulation of gut-associated lymphoid tissue with a vaccine or a challenge organism [5, 8]. The ELISPOT and ALS assays both utilize peripheral blood mononuclear cells (PBMC) that are collected about a week after antigen stimulation. In the ELISPOT assay, a technician or optical density reader enumerates the number of ASC in the peripheral blood that bind to specific antigen adhering to microtiter wells. Interpretation can be subjective and therefore a source of variability in this assay. In the ALS assay, PBMC are incubated in microtiter plates without antigen, and the amount of antibody that is spontaneously released into the supernatant is measured.

The ELISPOT assay has been widely used as a surrogate to indirectly measure intestinal immune responses to candidate vaccine antigens for a number of enteric vaccines including those directed at *Shigella* [6, 7, 9–11]. ELISPOT has higher sensitivity when done with fresh rather than frozen cells [10, 12], and this limitation can pose logistical difficulties in clinical trials. The ALS assay provides supernatants that are frozen, permitting validation by different users or laboratories. Since the supernatant collected in the ALS assay contains spontaneously released antibody, these supernatants can be tested for antibody against a potentially broader array of antigens and in different assay formats.

The ALS assay has been shown to be both sensitive and specific for mucosal infections or vaccinations with cholera [5], typhoid [10, 12], and enterotoxigenic *E. coli* [6, 13, 14], but has not previously been evaluated for use in *Shigella* studies. We investigated the use of the ALS assay as an alternative to ELISPOT for testing immune responses to *Shigella* in a clinical trial setting. The ALS method was both sensitive and specific for the detection of antibody responses against *Shigella* lipopolysaccharide (LPS), and ALS data correlated well with ELISPOT results.

2. Materials and Methods

2.1. Study Population

The study population for this analysis was composed of subjects from two previously published inpatient studies: one examining the safety and immunogenicity of a new oral, live attenuated *S. dysenteriae* type 1a vaccine (WRSd1) [15] and the other examining the efficacy of rifaximin as a prophylactic antibiotic for the prevention of shigellosis after challenge with *S. flexneri* 2a strain 2457T [11]. Our analysis included all 40 subjects who were given the WRSd1 vaccine and the 10 subjects who were administered placebo in the rifaximin prophylaxis study and were subsequently challenged with *S. flexneri* 2a.

2.2. ELISPOT assay

Ficoll-hypaque gradient centrifugation was used to isolate PBMC, and the number of ASC was determined by ELISPOT as previously described [6, 15]. Data were recorded as the number of spot-forming cells per 10^6 PBMC. A response was considered significant if there were ≥ 5 ASC per 10^6 PBMC.

2.3. ALS assay

PBMC were cultured using the ALS assay as described [6, 8]. The supernatants were rapidly thawed, and ELISA was performed as described [6]. A response was considered significant if there was a 3-fold increase in antibody titers from baseline. McKenzie et al [15] used a 4-fold rise in ALS antibody titer as the definition of a positive response based on the study protocol, but we used the more sensitive definition of a 3-fold rise in this comparative study.

2.4. Statistical Methods

Logistic regression models were used to determine the odds of having a significant antibody response according to ELISPOT given a significant response by ALS. The sensitivity, specificity, and percent correctly classified of the ALS assay were determined using ELISPOT as the comparator. Spearman correlation coefficients comparing ALS antibody responses to ELISPOT results were also used in order to test the strength of the relationship between assays. Responses were compared utilizing both binary (significant versus not significant titer fold-rise) and continuous (actual titer) data. All analyses were conducted using Stata 11 (StataCorp, College Station, TX).

3. Results

3.1. Summary of responses for subjects given WRSd1 or challenged with *S. flexneri*

A total of 50 subjects were analyzed, including 40 subjects vaccinated with *S. dysenteriae* 1 vaccine candidate WRSd1 and 10 placebo recipients from a rifaximin prophylaxis study subsequently challenged with *S. flexneri* 2a. Of the 40 subjects vaccinated with *S. dysenteriae* vaccine, 32 had a detectable immune response and of the 10 subjects challenged with *S. flexneri*, 9 had a detectable immune response (Table 1). Among the vaccinees, the IgA responses included 23 by the ALS assay and 24 by the ELISPOT assay and the IgG responses included 16 by the ALS assay and 7 by the ELISPOT assay (Table 1). In the 10 subjects challenged with *S. flexneri*, the IgA responses included 7 by the ALS assay and 7 by the ELISPOT assay (Table 1). The IgG responses included 5 by the ALS assay and none as measured by ELISPOT (Table 1).

3.2. Comparison of the frequency of immune response detected by the assays

3.2.1. Regression models—In a logistic model examining the odds of having a significant ELISPOT response given a positive ALS response in subjects in the *S. dysenteriae* vaccine study, the odds ratio was 11.4 for IgA ($p=0.001$) and 13.8 for IgG ($p=0.022$). For vaccinated subjects, the sensitivity, specificity, and percent correctly classified of the ALS assay (using the ELISPOT assay as the comparator) were 79%, 75%, and 78%, respectively for IgA antibody (Table 2). For IgG antibody the logistic model predicted ELISPOT results with 100% specificity among vaccinees, and 83% were correctly classified (Table 2). For subjects challenged with *S. flexneri*, the sensitivity, specificity, and percent correctly classified of the ALS assay were 86%, 67%, and 80%, respectively for IgA antibody responses (Table 2). Additionally, in a linear regression model of subjects in the WRSd1 trial the fold rise in ALS IgA titer had a significant linear correlation with ASC IgA counts, as well as fecal and serum IgA fold rises ($p < 0.001$) (data not shown).

3.2.2. Spearman correlation coefficients—In the *S. dysenteriae* vaccine study, comparison of all four antibody responses were statistically significant, suggesting that there is a strong correlation between the frequency of ALS and ELISPOT antibody responses (Table 3). Results for subjects challenged with *S. flexneri* were only statistically significant when examining IgA antibody levels in a continuous model (Table 3).

4. Discussion

The ALS assay detected immune responses in an equal or greater number of subjects than ELISPOT. Logistic regression models indicate that the presence of antibodies as detected by the ALS method greatly increase the odds of a subject having a positive ELISPOT response. Based on our observations from the *S. dysenteriae* vaccine and *S. flexneri* 2a challenge studies, the ALS assay was also found to be 79% to 86% sensitive at detecting IgA antibody responses when using ELISPOT as the comparator. It is important to note that comparable specificity was also found for IgG antibodies.

A recent WHO report recommended that ALS be considered the preferred method for studies of primary mucosal immune responses in ETEC studies [16]. The results of our analysis indicate that it may have similar utility for Shigella studies. The ALS assay is a reasonable alternative to ELISPOT given its apparent greater sensitivity in detecting mucosal immune responses and better objectivity given its reliance on an ELISA readout. ALS is also more practical for use in large clinical trials because supernatants can be frozen for later analysis, tested against a broader array of antigens, and transferred more easily between testing laboratories for verification.

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Highlights

- We compared ELISPOT and ALS for measuring immune responses to *Shigella* antigens.
- Responses measured by ALS are comparable to those measured by ELISPOT.
- ALS is a practical and flexible alternative to ELISPOT.

Number (percentage) of subjects with diarrhea, colonization, and mounting *Shigella*-specific immune responses by study and antibody assay^a.

Table 1

Number (%) with significant response/number tested	ALS (ELISA)		ASC (ELISPOT)	
	diarrhea	colonized	IgA	IgG
WRSD1 (n=40) (vaccinated)	8 (20)	9 (22.5)	23 (57.5)	16 (40)
			24 (60)	7 (17.5)
2457T (n=10) (challenged)	6 (60)	5 (50)	7 (70)	5 (50)
			7 (70)	0 (0)

^a A significant response was defined as: a 3-fold or greater rise in ALS titer from baseline; a minimum of 5 ASCs per 10⁶ PBMC and at least double the baseline value for ELISPOT.

Table 2

Sensitivity, specificity, and percent correctly classified of the ALS assay compared to the ELISPOT assay in terms of frequency of immune response.

Logistic Regression		Sensitivity (%)	Specificity (%)	Correctly Classified (%)
WRSd1 (n=40) (vaccinated)	IgA	79	75	78
	IgG	0	100	83
2457T (n=10) (challenged)	IgA	86	67	80
	IgG	NA	NA	NA

Table 3

Spearman correlation coefficients comparing the frequency of either a binary or a continuous immune response^a detected by the ALS assay to the frequency detected by the ELISPOT assay.

Spearman correlation coefficients		Binary coef. (p-value)	Continuous coef. (p-value)
WRSd1 (vaccinated) (n=40)	IgA	0.54 (<0.01)	0.67 (<0.01)
	IgG	0.43 (<0.01)	0.33 (0.04)
2457T (challenged) (n=10)	IgA	0.52 (0.12)	0.82 (<0.01)
	IgG	NA	0.47 (0.17)

A significant response was defined as: a minimum of 5 ASCs per 10 PBMC and at least double the baseline value for ELISPOT; a 3-fold or greater rise in ALS titer from baseline.