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Research Article www.enlivenarchive.org Enliven: Microbes and Microbial Techniques ISSN: 2576-2540

An Improved Wolbachia Surface Protein based ELISA

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Received Date: 28th November 2019
Accepted Date: 07th January 2020
Published Date: 08th January 2020

Citation: I. Ali, J. Seshu, R. Tamfu, and J. Chambers. An Improved Wolbachia Surface Protein based ELISA. Enliven: Microb Microbial Tech. 2020;7(1): 001-005.

Abstract

Introduction: Wolbachia bacteria are among the most successful and intriguing intracellular bacteria in nature. Bacterial surface proteins often function as antigens and host elicited antibodies constitute a sensitive means of detection. Detection of Wolbachia has relied on PCR amplification but PCR is not always a reliable means of detection due to primer specificity, strain diversity, degree of infection, and/or tissue sampled.

Objective: To develop a specific and sensitive ELISA for detection of host elicited IgG/IgM response against Wolbachia.

Methods: His-tagged recombinant Wolbachia Surface Protein (WSP) was expressed in *E. coli*, affinity purified, and validated by PAGE and Proteomic analyses. Rabbits were immunized with recombinant WSP and resulting antiserum affinity purified, and used in development of the ELISA.

Results: High titer IgG was detected in rabbits immunized with purified recombinant WSP. Qualitative assessment of 26 human serum samples revealed a mix of IgG and IgM. Sera from a variety but limited number of animals indicated a broad but positive IgG response.

Conclusion: A limited number of human and non-human sera were found to contain elicited anti-WSP antibodies. The ELISA reported here is a sensitive and potentially useful means of detecting elicited IgG/IgM in response to Wolbachia.

Keywords: Wolbachia; Surface Protein; ELISA.

Introduction

Wolbachia is a maternally inherited endosymbiont first discovered in 1924, and is among one of the most common life forms on Earth [1]. It is predominantly transmitted via females to developing eggs, but can also undergo horizontal transmission between host species [2]. Current estimates suggest that Wolbachia infects more than 60 % of all insect species but is also widespread in other invertebrates such as arachnids, crustaceans, and nematodes [3-5]. Although Wolbachia is generally thought of as a parasitic microbe which favors transmission at the cost of the host nuclear genome, it has evolved a wide range of host relations which play essential roles in various aspects of nutrition, reproduction, and pathogen resistance [6-8]. Although many studies have been conducted investigating Wolbachia's parasitic effect on the host, detection of host elicited WSP specific antibodies is limited to a few reports [9-11]. The availability of a more sensitive WSP antibody-based ELISA could be of value to the scientific community interested in this organism.

Materials and Methods

Recombinant protein expression and purification

His-tagged WSP cDNA coding for amino acids 25-236 (Accession # AJ252062.1) (GenScript, Piscataway, NJ), and subcloned into pET28A vector using NcoI and XhoI restriction sites. Following transformation of BL21 (DE3) cells, colonies were screened for recombinant protein expression. Briefly, cells were grown in LB-Kanamycin media at 37°C while shaking (150 oscillations/minute) to an OD_{600} of 0.5-0.6 followed by induction with 0.5 mM IPTG for an additional 3 hours. Cells were lysed in 50 mM Tris-HCL, pH 8.0 buffer containing 0.2 mg/ml lysozyme, 0.5 mM EDTA, 1mM PMSF, 5 mM β-Mercaptoethanol, and 250 mM NaCl on ice. Lysates were sonicated 3 times (30 seconds at 60% full power setting while on ice), and lysate material centrifuged at 10,000 rpm for 1 hour to pellet inclusion bodies. Inclusion bodies were washed twice with lysis buffer, and His-tagged protein extracted in lysis buffer contain-

ing 8M urea was applied to Ni-NTA Agarose beads, and eluted with buffer containing 250 mM imidazole for consistency. Protein was quantitated using the BioRad dye-binding assay with BSA as standard [12].

SDS-PAGE analysis

Electrophoresis was carried out under denaturing conditions according to the method of Laemmli [13]. Recombinant WSP (3.5 µg) was heated for 5 minutes at 95°C in the presence of SDS/β-mercaptoethanol, applied to a 10% BIS-Tris gel (Life Sciences Technology, Inc., Hewlett, NY), and proteins were electrophoretically separated at constant voltage (80 V) for 2 hours at room temperature. Gels were stained with Coomassie Blue.

Proteomic analysis

The gel band of interest, i.e., 26 kDa species (cf. Figure 1) was removed, reduced *in situ* with TCEP [tris-(2-carboxyethyl) phosphine], and alkylated in the dark with iodoacetamide prior to treatment with trypsin (Promega). Trypsin digests were analyzed by capillary HPLC-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) on a Thermo Fisher Orbitrap Fusion Lumos mass spectrometer fitted with a New Objective Picoview 550 nanospray interface.WSP digest 'online' HPLC separation was accomplished using a RSLC NANO HPLC system (Thermo Fisher/Dionex). Orbitrap acquired precursor ions (from 300-1500 m/z at 120,000 mass resolution), and data-dependent higher-energy collision dissociation (HCD) spectra were acquired at the same time in the linear trap using the "top speed" option. Mascot (Matrix Science) was used to search and compare spectra in a bacterial subset of the Uniprot database concatenated to a database of common protein contaminants. Cysteine carbamidomethylation was set as a fixed modification, and methionine oxidation, deamination of glutamine and asparagine, and acetylation of the protein N-terminus were considered as variable modifications. Trypsin was specified as the proteolytic enzyme with one missed cleavage allowed. Subset searching of the identified proteins was achieved by X! Tandem cross-correlation with Mascot results, and determination of WSP peptide identity probabilities was accomplished using Scaffold (Proteome Software). The threshold for acceptance of WSP peptide and protein assignments in Scaffold were 95 and 99%, respectively (peptide and protein FDR <0.1%). Mass spectrometry analysis was conducted in the Mass Spectrometry Core Laboratory of the Department of Biochemistry, the University of Texas Health Science Center at San Antonio.

Rabbit immunization

Adult, New Zealand White male (5-6 lbs) rabbits were used for production of anti-recombinant WSP antibody in a commercial USDA sanctioned facility. Rabbits were immunized with 100 µl (3.8 mg/ml) recombinant WSP antigen by subcutaneous injection (3-4 sites). Immunization was initiated on day 1 with Freund's complete adjuvant, and subsequent injections were performed at 2, 4, 6, and 8 weeks with Freund's incomplete adjuvant. A Pre-immune bleed was collected prior to immunization (day 0). Bleeds were collected at 3, 7, 9, and 11 weeks. Serum was separated at room temperature by centrifugation (15 minutes at 3,000 rpm), and stored at -20 oC until used.

WSP antibody purification

Recombinant WSP was coupled to Cyanogen bromide-activated Sepharose® 4B (Sigma Aldrich, St. Louis, MO) at a 1:3 (w/v) ratio. Cyanogen bromide (0.7 gm) dissolved in 0.7 ml DMSO was added to previously washed (distilled water) Sepharose gel suspended in 14 ml distilled water. Following gentle mixing (15 mins) of the DMSO-Cyanogen bromide-Sepharose gel suspension at room temperature,1.75 ml NaOH was added, and the required pH, i.e., 9-10 obtained. The reaction was terminated by washing with approximately 7 ml ice-cold distilled water. Following washing of the Sepharose gel with pH 9.6 carbonate buffer, recombinant WSP was added to the activated Sepharose gel at a 1:3 (w/v) ratio, and mixed overnight at 5°C.Following mixing, 400 µl 0.01 M Tris buffer, pH 8.0 was added, and the WSP-Sepharose coupled gel was stored at 5°C. Prior to affinity purification, WSP-Sepharose coupled gel was washed initially with 20 ml distilled water followed by a second wash with 20 ml 0.1M Glycine buffer, pH 2.5, and a final wash with 20 ml distilled water. Rabbit WSP antisera (20 ml) was passed over the WSP-Sepharose coupled gel column by gravity 3 times followed by washing with 1X PBS until absorbance at A_{280} was < 0.005. Bound antibody was eluted with 0.1M Glycine buffer, pH 2.5, and neutralized with 1.0 M Tris buffer, pH8.0. Affinity purified WSP antibody was dialyzed against 1X PBS twice at 5 °C(once for 3 hours, and once overnight), and served as source of immunoglobulins for ELISA development.

ELISA plate coating

A checkerboard titration was performed from 0.1 to 2 µg/ml, and 0.1 µg/ml was observed to be optimal. ELISA plates were prepared by coating recombinant WSP (100 µl/well = 10 ng) overnight at 4°C in PBS. Following washing with PBS containing 0.05 % Tween 20 (PBST) buffer 3X's, BSA blocking reagent was added to each well, incubated at room temperature for 1 hour after which time wells were aspirated using an ELISA wash apparatus, and subsequently dried for 3 hours before use.

ELISA

Detection of WSP specific elicited antibodies was carried out as follows: Affinity purified Rabbit anti-serum or human/non-human test sera (100 µl) was added to each well at a 1:100 dilution and incubated for 1 hour. Wells were washed 3 times with PBST wash buffer followed by addition of 100 µl Horseradish Peroxidase (HRP)-conjugated immunoglobulins (Alpha Diagnostics Intl., San Antonio, TX), e.g., goat anti-human IgG, goat anti-human IgM or goat anti-nonhuman species IgG specific, and incubated for 30 minutes at room temperature. Following washing (5 times) with wash buffer, 100 µl 3,3',5',5'-tetramethylbenzidine (TMB) substrate was added to each well, incubated for 15 minutes at room temperature, and reactions were terminated by addition of 100 µl 'Stop Solution' (1 % Sulfuric Acid). Absorbance at 450 nm was determined using an ELISA Plate reader (MPR-2100, Awareness Technologies,Westport, CT).

Quantitative assessment of recombinant WSP elicited IgG in immunized rabbits, i.e., whole rabbit antiserum was determined by comparison of A_{450} values post immunization to that of a

standard curve. Standard titer strips were generated as follows: Rabbit IgG (Equitech Bio., Kerrville, TX) was coated at specific concentrations, e.g., 3, 10, 30, and 100 ng, respectively. HRP-conjugate (100 µl Goat anti-nonhuman species IgG specific) was added to strips at appropriate dilution (1:200) for 30 minutes. Following washing (5 times), 100 µl TMB substrate was added to each well, and incubated for 15 minutes at room temperature.: Reactions were terminated with 100 µl 'Stop Solution' and absorbance at 450 nm determined. Absorbance for standards was linear from 3 to 100 ng. Quantitation of rabbit WSP specific elicited antibodies, i.e., IgG was determined using the recombinant WSP-based ELISA and absorbance (A_{450}) values within the linear range by appropriate dilution. Titer (µg/ml) was determined by multiplication of extrapolated A_{450} values by the dilution necessary to achieve A_{450} values within the standard linear range.

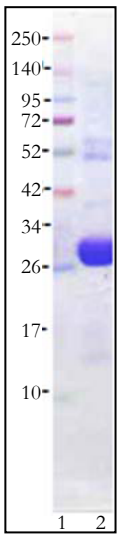
Results

His-tagged recombinant WSP was highly expressed in *E. coli* (10 mg/ml), and purified essentially to homogeneity. SDS-PAGE analysis revealed a single electrophoretic species of approximately 26 kDa under reducing/denaturing conditions (Figure 1), the identity of which was corroborated by mass spectroscopy/proteomic analysis (Data not shown). Immunization of animals with recombinant WSP produced high antibody titer from 3 to 9 weeks peaking at a titer greater than 1:1,000,000 (Figure 2), and served as source of immunoglobulin for affinity purification and ELISA development. Of twenty-six human serum samples qualitatively assessed for IgG and IgM using the ELISA (Figure 3A), approximately 30 and 60 % tested positive for IgM and IgG, respectively. Qualitative assessment of IgG in non-human sera indicated cat to be lowest, i.e., ~ 13 % followed by mice (~ 57 %), and camel (70 %) with remaining sera ranging from 80-100 % (Figure 3B).

Discussion

Popovici et al., using a Wolbachia whole cell extract coating antigen based ELISA derived from *Aedes aegypti* cells reported no statistically significant difference in anti-Wolbachia IgG when compared to controls [9]. However, whole cell-based assays are generally less sensitive due to impurities in the preparation. Alternatively, Punkosdy and co-workers detected WSP antibodies in rhesus monkeys infected with *Brugia malayi* [10], and human serum from patients with Lymphatic Filariasis [11]

Figure 1. SDS-PAGE analysis.



SDS-PAGE analysis was carried out as described under 'Materials and Methods'. Lane 1, Standard ladder (kDa); Lane 2, recombinant WSP (3.5 µg).

Figure 2. WSP immunogenicity

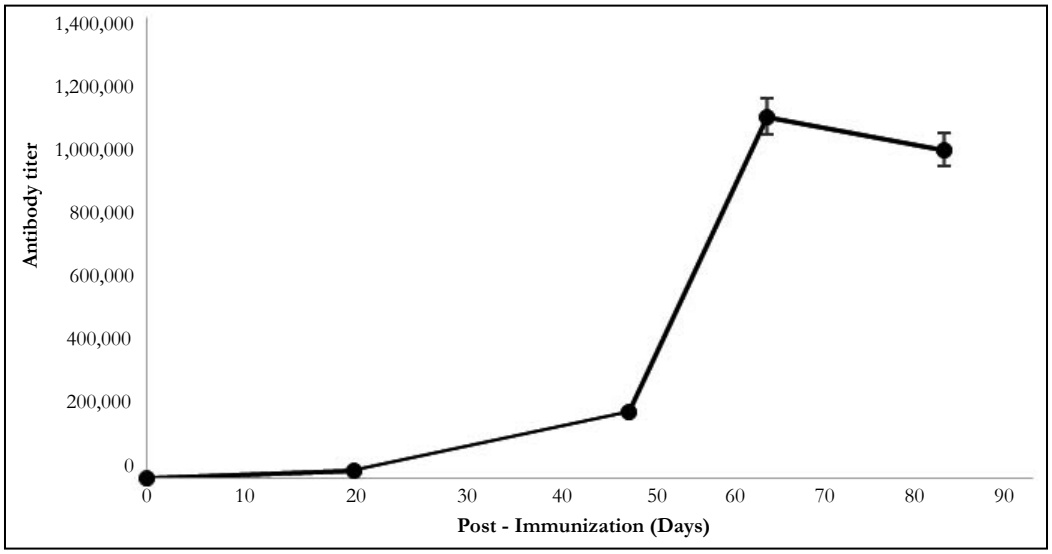
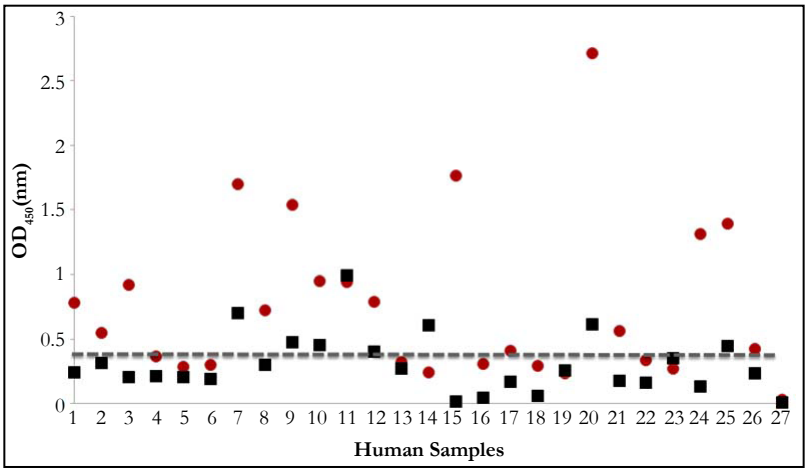


Figure 2. Wolbachia IgG titer was determined as described under 'Materials and Methods'.

Figure 3. Qualitative assessment of WSP elicited antibodies in various sera.



A

Animal	Above positive cut-off	Total Animals	% Positive
Rabbit	5	5	100%
Pre-immune rabbit serum	0	5	0%
Bats	5	5	100%
Mice	4	7	57%
Camels	7	10	70%
Cats	1	8	13%
Dogs	8	8	100%
Chickens	7	8	88%
Bovine	12	15	80%
Pigs	12	12	100%

B

Figure 3: WSP antibodies were detected by ELISA as previously described under 'Materials and Methods'. Frame A: Human serum samples (n = 26) were screened for anti-WSP IgG (circles) and IgM (squares) in duplicate (average of replicates). Sample 27 is the blank. The grey line represents the threshold 'cut-off' value of 8 human serum samples which gave the lowest A_{450} values (mean value = 0.28 plus 2 standard deviations, i.e., 0.03) at a dilution of 1:100. Values above the cut-off were considered positive while values below were considered negative. Human sera were collectively obtained from various FDA sanctioned laboratories. Frame B: Nonhuman sera. Negative values were assigned using the same threshold 'cut-off' derived for human samples. All non-human sera were obtained from commercial sources and are of unknown origin.

using recombinant WSP and biotinylated mouse antihuman IgG which was detected using streptavidin conjugated Alkaline Phosphatase mediated hydrolysis of *p*-nitrophenyl phosphate (PNPP). The assay reported here differs in that the means of detection, i.e., HRP-TMB coupled reduction of H_2O_2 has a lower limit of detection (~7-8 pg/ml) which is greater than 3-orders of magnitude more sensitive than the hydrolysis of PNPP (lower limit of detection ~ 10 ng/well). Additionally the amount of recombinant WSP antigen required per well, i.e., 10 ng is considerably less than that required in the Punkosdy assay, i.e., 50-200 ng.

The ELISA reported here detected recombinant WSP antigen bound antibodies from human and several non-human sera. The majority of non-human sera screened were very positive for IgG with the exception of cats, i.e., ~13 %. It is interesting to note that all but one non-human serum, i.e., rabbits used in this study was obtained from commercial sources and of known origin, and as such have not encountered Wolbachia thus testing negative as indicated by pre-immune rabbit serum. In comparison, other animals that possibly resided in the "wild" having a diet comprised of plants and insects in which Wolbachia is a native endosymbiont should elicit an immune response, and thus test positive.

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