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RESEARCH ARTICLE

Rapid identification of *Shigella sonnei* by the use of specific monoclonal antibody based dot ELISA

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Abstract

Development of a simple system for the rapid and specific detection of pathogenic strains of *Shigella sonnei* is of great importance as outbreaks associated with *Shigella sonnei* have been reported frequently. In the present study, monoclonal antibodies (MAbs) were raised against outer membrane protein of *Shigella sonnei*. A set of three stabilized hybridoma cell lines were generated. MAb Ss 33 exhibited specific reaction to the whole cells of *Shigella sonnei* strains. The other two MAbs, Ss 2 and Ss 4 had cross reactions with other organisms. The MAb Ss 33 did not show any cross-reaction with whole cell lysate of *Salmonella typhimurium, Escherichia coli* and other species of *Enterobacteriaceae* in plate ELISA and Western blot analysis. MAb Ss 33 was further used in the development of a simple dot ELISA system for the specific detection of *S. sonnei.* The presence of *S. sonnei* recovered from clinical, and diverse samples were evaluated by this dot ELISA and results were compared with the conventional biochemical, serological methods and also by in-house developed PCR. Data suggests this newly developed dot ELISA method have the potential for their use in reliable, rapid detection of *Shigella sonnei* at a relatively low cost.

Introduction:

Shigella spp. is a virulent bacterium of *Enterobacteriaceae* family. Shigellosis is an acute gastroenteritis caused by Shigellae, including *Shigella dysenteriae, Shigella flexneri, Shigella boydii* and *Shigella sonnei*. The most common *Shigella* species in developed countries is *Shigella sonnei* (77 percent) followed by *Shigella flexneri* (16 percent), *Shigella boydii* (2 percent) and *Shigella dysenteriae* (1 percent) (Wen-Bin Hsu et al., 2007). *Shigella sonnei* is a non-motile, nonspore-forming, facultative anaerobic Gram-negative bacterium causing enteric infectious disease Shigellosis in both developed and developing countries and has been the most common cause of endemic disease in developing countries. A major food-borne threat to public health in many developed countries where the issues of sanitation are concerned (Thomas and Gerald 2000; Niyogi SK 2005; Sansonetti 2006). *S. sonnei* is the predominant species in developed countries, one of the most frequent in the tropics and *S. sonnei* has emerged as a major global cause of Shigellosis (Vartul sangal et al., 2013; Preston and Borczyk 1994; Heier et al., 2009). *S. sonnei* is spread mainly by means of faecal-oral transmission. Other possible modes of transmission can be from ingestion of contaminated food or water and subcutaneous contact with inanimate objects. *S. sonnei's* infectivity dose is very low; as few as 100-200 bacteria are needed to cause a clinical infection, Shigellosis (Shiferaw et al., 2004; Stothard et al., 2005). *S. sonnei* infection is mainly characterized by invasion of the intestinal mucosa. Invasion is mediated mainly by Invasion Plasmid Antigen (Ipa) protein and by outer membrane proteins (Voahangy Rasolofo-Razanamparany et al., 2001). The outer membrane proteins are involved in the formation of protruding pores at the colonic epithelial cell wall in order to invade other neighboring cells in the colon which facilitates intra and intercellular movement of bacterium. Conventional methodology for identifying *S. sonnei* is based on isolation

in pure cultures, biochemical and serological tests. The epidemiological investigation of *S. sonnei* is hampered by the lack of a rapid identification method, taxonomic confusion and the limitation of conventional methods. This conventional methodology requires a substantial amount of time to be carried out and is expensive and less sensitive (Islam et al., 1998; Kenia et al., 2010). Detection systems like PCR formats for the rapid detection of virulence genes associated with *Shigella* spp. require prior isolation of bacterial DNA, preparation of enzyme reaction mix, and expensive instruments for nucleic acid amplification and sophisticated laboratory facility. Fewer immunoassays such as ELISA tests use polyclonal or monoclonal antibodies or a combination of both (Warren et al., 2006). However, the use of polyclonal antibodies in these tests may produce a high number of false positive results due to cross-reactions of shared epitopes present in other bacteria or even in food components. Moreover, discriminating among *Shigella* species is of value in epidemiologic studies (Sur et al., 2004). Therefore, there is a need for generating relevant monoclonal antibodies that can lead to the development of low cost, simple to use, yet rapid, test systems or kits for confirmed identification of *Shigella* species recovered from food or clinical samples under field conditions. In the present invention, we report on the generation of specific monoclonal antibody against *S. sonnei* utilizing outer membrane proteins (Omp) as source of antigen. In addition, we report, the application of this specifically generated monoclonal antibody for use in a simple, reliable, field use, low cost dot ELISA system for the specific detection of *S. sonnei* from food or clinical samples.

Materials and methods:

Bacterial strains and culture conditions:

The bacterial strains used in this study are listed in Table 1. Reference strains were obtained from American Type Culture Collection (Hi-Media, Mumbai), SDM Medical College (Dharwad, India), JSS Medical College (Mysore, India). The isolates of *Shigella sonnei* used in this study were recovered from clinical samples, water, raw meat and poultry. All the media used in this study were purchased from Hi-Media labs, Mumbai. The *S. sonnei* strains were grown on tryptic soy broth (TSB) and plated on Salmonella Shigella agar plates. The plates were incubated at 37 ^{0} C for 24 h. Characteristic colourless colonies were selected and maintained in 15% glycerol stocks at -80^{0}C. All the other cultures of *Enterobacteriaceae* were grown in tryptic soy broth and incubated at 37^{0}C for 18 h. For molecular biology experiments, the bacteria were cultured in Luria Bertani broth.

Preparation and purification of Outer membrane protein:

Outer membrane protein (Omp) was extracted from the standard strain of *S. sonnei* by employing modified method of Sachan and Agarwal (2002). Briefly, *S. sonnei* was grown overnight in 200 ml of LB broth. The cells were recovered by centrifugation at 5,000 rpm for 30 min at 4^{0}C and were resuspended in 3 ml of 10 mM tris amino methane buffer containing 0.3 percent NaCl (pH-8.0). Cells were frozen at -70^{0}C for 30 min followed by thawing at 60^{0}C for 15 min for six times to lyse the cells and centrifuged at 10,000 rpm for 2 min to remove the cell debris and unlysed cells. The supernatant was transferred to new tubes and centrifuged for 1 h at 17,000 rpm at 4^{0}C. Resulting pellets of cell envelop suspensions were incubated overnight at 4^{0}C with 3 percent Sodium lauroyl sarcosinate (sarkosyl) in 10 mM tris buffer. Outer membrane protein was obtained by centrifugation at 17,000 rpm for 1 h.

Immunization of the Mice:

Balb/c mice (6 weeks old, male) were immunized with purified outer membrane protein intramuscularly. Primary immunization of each animal was done with 50 µg of purified outer membrane protein in Freund's complete adjuvant (Sigma, India). Subsequent two booster doses of 10 µg protein in Freund's incomplete adjuvant (Sigma, India) were given 14th and 28th day. Antibody titers were measured by plate-ELISA (Engvall and Perlman, 1971). Hyperimmune serum obtained after the last boost was used in Western immunoblot analysis as described below.

Murine hybridomas and MAbs:

Immunized Balb/c mice were sensitized with three successive intraperitoneal injections and sacrificed humanely. The study was conducted under an institutional committee approved protocol ethical aspects. The sensitized splenocytes were fused with SP2/0-Ag-14 (ATCC® CRL-1581™) mouse myeloma cell line following the method of Kohler and Milstein (1975) with minor modifications such as performing fusion of cells without lysis of RBCs. The rate of myeloma cells to splenocytes was maintained at 3:1 at the time of fusion and immediately after fusion the cells were held for 2 min before adding growth media.

SDS-PAGE and Western Blot analysis:

SDS–PAGE was performed by the method of Laemmli (1970). The samples prepared using outer membrane protein of *Shigella sonnei* and crude protein preparations and other organisms in 2X lysis buffer (0.0625 M tris, 2 percent SDS, 10 percent glycine, 5 percent 2-mercaptoethanol, 0.001 percent bromo-phenol blue, pH 6.8) were electrophoretically separated in a 10 percent separating gel and 3.6 percent stacking gel in the presence of 0.5% SDS at a constant current of 15 mA per gel for 3 h in an electrophoretic cell [BioRad's mini gel apparatus containing electrode buffer (pH 8.6)]. The separated polypeptides were transferred onto 0.45 µm pore size nitrocellulose membrane (Millipore, USA) by the method described by Towbin et al., (1979) using 25 mM tris hydrocloride-192 mM glycine buffer (pH 8.3), containing 20% methanol at a constant voltage of 45 v for 1 h in a transblot cell (BioRad transfer apparatus, USA). The free sites of the nitrocellulose membrane were saturated by incubating in 5 percent skimmed milk powder in PBS, overnight at 4^0C. Membranes were washed three times with washing buffer PBST (phosphate buffered saline with 0.05 percent tween 20), each with shaking for 5 min and incubated with monoclonal antibodies. Following further three washings in PBST, incubation was carried out with anti-mouse IgG HRP conjugate (Sigma chemicals, USA) diluted at 1:1,000 in PBS at 37^0C for 1 h. After three washings of 5 in each in PBST, the nitrocellulose membranes were developed with DAB-H_2O_2 substrate. The enzyme reaction was terminated by washing the sheet in 0.1 M H_2SO_4.

Indirect Plate Enzyme Linked Immunosorbent Assay:

The indirect plate ELISA was performed as per the method described by Engvall and Perlman (1971) with suitable modifications. Omp preparation of *S. sonnei* strains and soluble extracts of sonicated antigens of other species of the family *Enterobacteriaceae* as mentioned in Table 1. 100 µl volumes were added to the microtiter wells and blocked with 5 percent bovine serum albumin (BSA) in phosphate buffered saline (PBS). Later incubated with monoclonal antibody for 30 min. Subsequently incubated with secondary antibody labelled with enzyme (goat anti-mice IgG-HRP, 1:1,000) for 30 min. After washing with PBST, enzyme substrate reaction was visualized by using the OPD-H_2O_2 substrate.

Dot Enzyme Linked Immunosorbent Assay:

Dot ELISA was standardized by spotting the cells in carbonate and bicarbonate buffer (10 mM) directly onto the nitrocellulose membrane and the cells were fixed by hot air drying. The membrane was later blocked by 5 percent bovine serum albumin protein (BSA) in phosphate buffered saline (PBS). Later incubated with monoclonal antibody for 30 min. Subsequently incubated with secondary antibody labelled with enzyme (goat anti-mouse IgG-HRP, 1:1,000) for 30 min. After washing with PBST, enzyme substrate reaction was visualized by using the commercially available substrate TMB/H_2O_2.

Sensitivity of MAbs by dot ELISA:

Sensitivity of the dot ELISA method standardized was checked by spotting different cell concentrations of *S. sonnei* i.e., from 10^8 to10^1 CFU/ml were coated onto the nitrocellulose membrane and cells were fixed, blocked by 5 percent BSA in PBS. Later incubated with monoclonal antibody for 30 min. Subsequently incubated with secondary antibody labelled with enzyme (goat anti-mouse IgG-HRP, 1:1,000) for 30 min. After washing with PBST, enzyme substrate reaction was visualized by using the commercially available substrate TMB/H_2O_2.

Characterization of MAbs:

Subclasses of monoclonal antibodies (immunoglobulins) were identified by isotyping, which was carried out using commercial antibody isotyping kit (ISO2, Sigma, India), following manufacturer's instructions.

PCR amplification:

Preparation of DNA template and PCR:

DNA was extracted from the overnight cultures of bacteria grown in LB broth. Template DNA from each bacterial strain was purified by boiling method (Theron et al., 2001). One milliliter of culture broth was removed and centrifuged at 10,000 rpm for 3 min. The pellet was resupended in 50 mL of distilled water, boiled for 12 min, and centrifuged again for 3 min at 12,000 rpm. The supernatant was transferred to a new microcentrifuge tube and stored at -20^0C for use.

Oligonucleotides and PCR:

Two sets of oligos were designed to detect the *osp C1* (Outer Shigella protein) and *ipaH* (invasive plasmid antigen H) genes using the GenBank database sequences (Table 2). Genus specific regions were selected and primers were designed with Gene Runner software (Hastings Software, Inc., Hastings, NY). To check the presence of inhibitors within PCR mixture, an IAC was constructed. The primers used in this reaction had 5' overhanging ends, which were identical to the primer *osp C1* (Outer *Shigella* protein), whereas 3' ends were complementary to a DNA sequence of pUC 19 (Table 2). All primers used in the study were procured from Eurofins Biotech Pvt. Ltd., Bangalore, India. PCR products ranged from 325 bp to 645 bp in length.

Construction of IAC DNA:

The PCR reaction mixture for generation of IAC DNA contained 1.0 µmol l^{-1} of each primer, 0.2 m mol l^{-1} each dNTP (MBI Fermentas, Canada), 0.5 units of *Taq* polymerase, 2.0 mmol l^{-1} $MgCl_2$ in 1 X PCR buffer (MBI Fermentas, Canada) with 350 pg of template DNA. The reaction procedure consisted of 30 cycles of denaturation at 94^0C for 1 min, primer annealing at 57^0C for 1min and extension at 72^0C for 1 min. The DNA was denatured for 4 min in the beginning and finally extended for 10 min at 72^0C (Eppendorf master cycler gradient, Hamburg Germany). PCR product was purified using commercially available kit (Sigma Co.). The concentration of IAC DNA was determined spectrophotometrically at 260 nm and was stored in DDW at -20^0C.The following equation was used to calculate the copy number of the PCR product concentration: weight of PCR fragment (in g $\mu l^{-1)}$ X ($6.023X10^{23}$) / (660 $gmoll^{-1}$ X number of base pairs of PCR fragment) = the number of genomic copy per microlitre (Kumar et al., 2006).

Triplex PCR was carried out in 30 µl reaction containing 0.4 µmol l^{-1} of *osp* F and osp R, 0.3 µmol l^{-1} of ipa H F and R primers, 0.2 mmol l^{-1} of each dNTPs, 1.2 units of *Taq* polymerase, 1.6 mmol l^{-1} $MgCl_2$ in 1X PCR buffer (MBI Fermentas) with 1.0 µl of template DNA. Various concentrations of IAC DNA were tried before choosing 10^4 copies per reaction. Amplification consisted of initial denaturation at 94^0C for 5 min followed by 30 cycles of denaturation at 94^0C for 1 min, primer annealing at 57^0C for 2 min and extension at 72^0C for 2 min and a final extension at 72^0C for 10 min followed. The PCR products were analyzed on a 2 percent (w/v) Agarose gel (Fig. 6).

Sensitivity of triplex-PCR:

To assess the minimum amount of *S. sonnei* DNA detectable by triplex-PCR, tenfold serial dilutions of overnight grown culture of *S. sonnei* was serially diluted at a concentration of 10^6–100 CFU ml^{-1}. DNA was prepared by boiling method as described earlier. A 1.0 µl aliquot of each dilution was added to five separate PCR tubes. *E. coli* genomic DNA was used as a negative control.

Specificity of triplex-PCR:

The specificity of the triplex-PCR primers was determined against different organisms as shown in Table 1 by taking 1 µl of template DNA from each of the organism.

Results:

Production of monoclonal antibodies:

Splenocytes from immunized mice showing the highest serum antibody titers were used for the fusion experiments. A total of 3 hybridoma cell lines, designated Ss 33, Ss 4 and Ss 2 were identified following single splenocyte–myeloma fusion and screening the resultant hybridoma supernatants for antibodies to capture *Shigella sonnei*. For further characterization of the cell lines, antibody-containing supernatants were tested for reactivity with the culture free supernatants as test antigens in Western blotting.

Characterization of monoclonal antibodies:

Isotyping analysis demonstrated that the MAbs Ss 33 belonged to IgG3 class and the other two Ss 4 and Ss 2 were IgG2a in nature (Fig.1).

Specificity of monoclonal antibodies by indirect ELISA:

MAb Ss 33 exhibited specific reaction to the omp and whole cell lysate of *S. sonnei*. All the standard strains and isolates of *S. sonnei* showed an OD above 0.75, whereas the OD values obtained with other species of *Shigella* and other members of *Enterobacteriaceae* were found much below the cut off value. However, the other two MAbs, Ss 2 and Ss 4 showed cross reactivity with protein A of *S. aureus* and also with other members of *Enterbacteriaceae* family.

Specificity of MAbs by Dot ELISA:

Whole cell lysates of cultures of *S. sonnei*, other species of *Shigella boydii*, other members of *Enterobacteriaceae* and other important food borne pathogens (Table 1) were subjected to dot ELISA test. The result is provided in Table 1 and Fig 3. In case of MAb Ss 33, positive signals were obtained only for strains of *S. sonnei*, ATCC 25931 and *S. sonnei* Dharwad. No signals were obtained for other species of *Shigella* such as *Shigella boydii* and *Shigella flexneri* Dharwad; and microorganims of other genera with proving its high specificity for *Shigella sonnei* only. However, the other two MAbs showed cross reactivity with protein A of *S. aureus* and with other members of *Enterbacteriaceae* family (Fig 4 and 5).

Sensitivity of MAbs by dot ELISA:

The dot ELISA method standardized with MAb Ss33 was found to be highly sensitive and signals were observed even with the cell concentration as low as 10^2 CFU/ml suggesting the high sensitivity of the method (Fig. 6).

Specificity of MAbs by Western blot analysis:

Subsequent Western blot results also demonstrated the specificity of the MAb Ss 33 which reacted specifically with *S. sonnei* only (Fig.2). Whereas, cross reactivity was observed with the other two MAbs.

Standardization of duplex PCR with IAC:

A duplex PCR with an IAC was also standardized to confirm the specificity of monoclonal antibodies. Fig 7. shows the presence of amplified products after agarose gel electrophoresis. Reliable amplification of three bands of *osp* C1, *ipa* H, and IAC were obtained. The mPCR format was assessed on reference (ATCC 25931) strains of *Shigella* spp. and also on the isolates recovered from food and environmental samples. The *Shigella* spp. having been identified by the conventional biochemical and serological tests were unequivocally detected positive by the newly developed triplex PCR format.

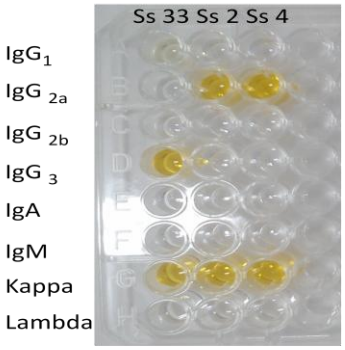

Fig. 1. Isotyping of Monoclonal antibodies raised against *Shigella sonnei* OMP

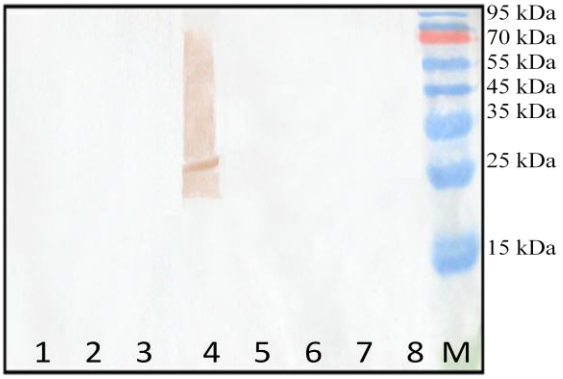

Fig. 2 Western blot analysis of Mab Ss 33 with Whole cell lysate of different organisms

Lane1: WCL of *S. flexneri* ; Lane 2: WCL of *S. boydii*; Lane 3: WCL of *S. boydii* ; Lane 4: WCL of *S. sonnei* ; Lane 5: WCL of *E. coli*; Lane 6: WCL of *S. enterica* Serovar Typhimurium ; Lane 7: WCL of *C. freundii* ; Lane 8: WCL of *S. aureus* ; M: Mol. Wt marker

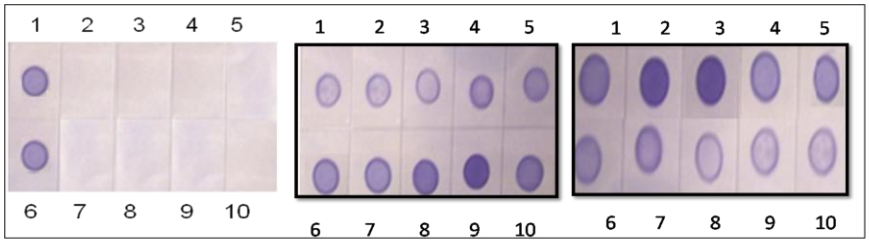

Fig. 3. Mab Ss-33 **Fig. 4. Mab Ss- 4** **Fig.5. Mab Ss-2**

Fig. 3-5. Characterization of Monoclonal Antibody Ss-33, Ss-4 and Ss-2 by Dot ELISA

1: *S. sonnei* ATCC 25931 omp; 2: *E. coli* ATCC 10536; 3: *S. enterica* serovar Typhimurium ATCC13311; 4: *S. boydii* ATCC 9207; 5:*S. flexneri* ATCC 12022; 6:*S. sonnei* ATCC 25931 cells; 7:*Klebsiella* sp AIIMS ; 8:*A. hydrophila* G47; 9: *Citrobacter freundii* Dharwad;10: *S. aurues* NCIM 2120.

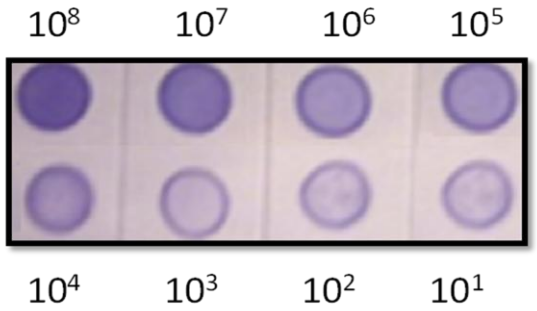

Fig. 6. Sensitivity testing of Mab Ss- 33 against different cell concentrations of *S. sonnei*

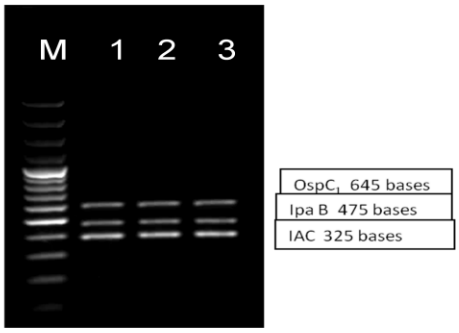

Fig. 7. Triplex PCR format for the detection of *Shigella* spp.

Sl No.	Test organism	*S. sonnei* Omp MoAb Ss-33				
		Western Blot	Plate ELISA	Dot ELISA	PCR	
					ospC	*ipa H*
1.	***Shigella boydii* ATCC 9207**	-	-	-	+	+
2.	***Shigella flexneri* Dharwad (SDM)**	-	-	-	+	+
3.	***Shigella sonnei* ATCC 25931**	+	+	+	+	+
4.	***Shigella dysentriae* Dharwad (SDM)**	-	-	-	+	+
5.	***E. coli* ATCC 10536**	-	-	-	-	-
6.	***E. coli* MTCC 40**	-	-	-	-	-
7.	***Salmonella typhimurium* ATCC 13311**	-	-	-	-	-
8.	***Salmonella typhimurium* KMC – 1**	-	-	-	-	-
9.	***Proteus mirabilis* AIIMS**	-	-	-	-	-
10.	***Citrobacter freundii* Dharwad**	-	-	-	-	-
11.	***Y. enterocolitica* 3099**	-	-	-	-	-
12.	***Enterobacter aerogenes* Dharwad**	-	-	-	-	-

13.	***Klebsiella* sp AIIMS**	-	-	-	-	-
14.	***B. cereus***	-	-	-	-	-
15.	***A. hydrophila* G47**	-	-	-	-	-
16.	***S. sonnei* Dharwad**	+	-	+	+	+
17.	***S. aureus* NCIM 2120**	-	-	-	-	-
18.	***L. monocytogenes* ATCC 19111**	_	_	_	-	-

Table 1. Evaluation of monoclonal antibody Ss-33 against other organisms by Plate, Dot and Western blot analysis

Table 2. List of genes targeted and primer sequences

Gene	Sequence	Base pair	Accession number
IAC For	CACTTGAGCACCTCGATAAGCCAGTCGGGAAACCTGT	325 bps	L09137.2
IAC Rev	CCAGACTAACAGGATCAAACCTCTGACTTGAGCGTC		
*OspC1*For	CACTTGAGCACCTCGATAAG	645 bps	AB819726.1
*OspC1*Rev	CCAGACTAACAGGATCAA		
IpaB For	GTCTCAGCAACAGGCAAG	465 bps	AF330480.1
IpaB Rev	CCAACTAGTTGAATAAAGG		

Discussion:

Worldwide, *Shigella* is estimated to cause 80–165 million cases of disease and 6,00,000 deaths annually. In US, 70 percent of Shigellosis episodes are caused by *S. sonnei*. Previously, *S. sonnei* has been chiefly responsible for dysentery in developed countries (Claudia et al., 2013; Suvash Chandra Ojha et al., 2013; Abu et al., 2013); however, in recent years, *S. sonnei* has become the dominant serotype causing Shigellosis in developing countries as well (Qu et al., 2012; Holt et al., 2012). Moreover, prevalence of multiple drug resistant strains of *S. sonnei* is increasing alarmingly during the past decade (Sivapalasingam et al., 2006). Therefore, it is crucial to develop a rapid method for identifying the *S. sonnei* in order to limit and control outbreaks. Classical methods though are the gold standards are time consuming and labor intensive and have low sensitivity. Moreover, these methods cannot differentiate the species of the genus *Shigella*. Molecular methods like PCR assays can overcome some of the shortcomings of culture methods but requires instrumentation, DNA extraction and electrophoretic separation to visualize the amplicons.

To overcome these drawbacks of existing techniques, we developed a dot ELISA method and evaluated its ability to detect *S. sonnei*. The use of antibody is a powerful diagnostic tool against bacterial infections. Advances in hybridoma technology have led to the development of a large variety of monoclonal antibody molecules for research, diagnosis and therapy. In the present study, we report on the generation of specific monoclonal antibody molecules against *S. sonnei* utilizing outer membrane proteins (Omp) as source of antigen. A total of three monoclonal antibodies Ss2 (IgG2a), Ss4 (IgG2a) and Ss33 (IgG3) were identified after screening for the ability of hybridoma supernatants to bind specifically with *S. sonnei*. From among the monoclonal antibodies generated and tested for specificity using indirect plate ELISA, monoclonal antibody Ss-33 had specific reaction to *S. sonnei*

ATCC 25931 in indirect plate, dot ELISA and also in Western blot analysis. No reaction was seen against *E. coli*, *S. flexneri*, *S. boydi*, *Klebsiella*, *S. typhi*, *S. typhimurium*, *Proteus* sp, *Citrobacter*, *S. aureus*, *B. cereus*, *A. hydrophila*, *Y. enterocolitica* and other relevant food borne pathogenic bacteria (Table 1). (Fig.2 and 3) The other two monoclonal antibodies showed reaction with other organisms other than *S. sonnei*. Conditions were standardized to utilize monoclonal antibody Ss-33 for the direct detection of the organism by a simple dot ELISA method. The infective dose of *S. sonnei* is as low as 10 – 100 organisms (Stothard et al., 2005; Kenia et al., 2010) and the presently described dot-ELISA showed a better degree of sensitivity and it showed signals even with the cell concentration as low as 10 cells (Fig. 6). Evaluation of the newly developed dot ELISA system was carried out with 150 food, clinical and environmental samples and also with the reference strains. Seven *S. sonnei* isolates were obtained from 73 diarrheal samples collected from various hospitals. Simultaneously, all the isolates were also characterized by the in house developed triplex PCR, conventional biochemical tests followed by serology for species level identification [commercial antisera (Denka seiken co. ltd, Japan)]. Seven isolates which were confirmed as *S. sonnei* by serology and other biochemical tests were unequivocally confirmed as *S. sonnei* by newly developed dot ELISA system. The primers for the amplification of *ipaH* and *ospC1* gene along with an IAC for the duplex PCR format reported here, were designed with the view to have a common annealing temperature to get preferable amplification at a single temperature and care was also taken to maintain at least 80 bps difference between product sizes for good resolution during agarose gel electrophoresis. An IAC was also included to rule out false results. According to guidelines for Molecular Diagnostic Methods for Infectious Diseases (MM3-A2, 2006), incorporation of an internal control in the reaction is essential for the diagnostic test to exclude false negative result or the presence of inhibitors. The triplex PCR format developed in the study is sensitive, simple, specific and can be used for simultaneous and rapid detection of all the four species of the genus *Shigella* from food and environmental samples (Fig. 7). The isolates which were identified as *Shigella* spp. by biochemical tests were unequivocally detected positive by the in-house developed triplex PCR format. The dot ELISA test we developed and evaluated is rapid, simple to readout, able to be used by minimally trained personnel, without the need for specific laboratory reagents or specialized laboratory equipment and has the ability to function at ambient conditions. This technique is more economical and practical than the traditional methods or the molecular assays.

In conclusion, specific detection of *S. sonnei* in field conditions still remains difficult. When compared to conventional biochemical, serological and other techniques like PCR, the dot ELISA method described in the present investigation for the species specific identification of *S. sonnei* by involving MAb Ss 33 can be of immense help during the frequently emerging Shigellosis, an endemic disease throughout the world caused specifically by *Shigella sonnei*. In-time treatment requires correct and rapid diagnosis of the causative organism.

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