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PREPARATION AND CHARACTERIZATION OF ANTIGENS AGAINSTISOLATED *Shigella spp* FROM INFECTED STOOL SAMPLES

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ABSTRACT

Shigellosis, also known as bacillary dysentery or *Shigella* dysentery, is an enteric bacterial infection caused by a group of *Shigella spp*. Nowadays, shigellosis is still a majorhealth problem in many parts of world, especially in the developing countries. Bacteria of the genus *Shigella spp* are human pathogens that infect the gastro-intestinal tract and cause acute shigellosis. *Shigella spp* are Gram-negative, non-motile, facultative anaerobic pathogens that are closely related to *Escherichia coli* but have evolved specific traits of pathogenicity, physiology and serology. *Shigella flexnari* strains were isolated and characterized by various isolation and identification methods.

KEYWORDS: *Shigella spp*, ELISA, IgY.

1. INTRODUCTION

Shigellosis, an acute invasive enteric infection caused by *Shigella* spp., is recognized as a major public health problem (WHO 2005). They are also common especially with foods that are subjected to processing or preparation by hand, are exposed to a limited heat treatment or are served raw to the consumer (Wu, *et al*.2000). Despite the high incidence of shigellosis, there is limited data on the prevalence of *Shigella* spp. amongst food handlers or on food products. Most of the time, they are usually confirmed by clinical isolates before the contaminated food is found. Several protocols have been developed for direct detection of

Shigella spp. in fecal, food and environmental samples to overcome some of the short comings of conventional culture methods. In this context, current application of PCR techniques to diagnosis *Shigella spp*.

Shigellosis is a leading cause of human diarrheal disease. Each year millions of cases occur, particularly in developing countries, with over 1 million cases resulting in death. The constant emergence of antibiotic resistance in *Shigella spp*. even to the newest antibiotics, underscores the need for an effective vaccine to help control *Shigella* disease. Vaccine strategies must consider the need for protection against four species of *Shigella* (*S. flexnari, S. sonnei, S. dysenteriae,* and *S. boydii*) with over 45 different serotypes, and also enter invasive *Escherichia coli* (EIEC), as cross-protection is not significant between the species. The pathogenesis of *Shigella* is attributed to the organism's ability to invade, replicate intracellular, and spread intercellular within the colonic epithelium. The invasion of host cells by *Shigella spp*. is a complex multifactorial event in which many different bacterial proteins are involved.

In the present study, there is no animal reservoir for *Shigella spp*, and infection is transmitted person-to-person, via fomites, and from ingestion of contaminated food or water. Shigellosis is therefore associated with poor sanitation and hygiene and limited access to clean drinking water. Healthy individuals with mild infections usually recover without specific treatment, but because *Shigella spp* invades the mucosal lining of the colon, it often causes dysentery, which is not amenable to oral rehydration. Antibiotic treatment is recommended for dysentery, severe shigellosis, and individuals with compromised immune systems. However, the emergence of multi-drug-resistant strains of *Shigella spp* further complicates antibiotic treatment, making prevention of infection critical. In addition, due to the number of other enteric pathogens that also affect children in early life, another important goal for a *Shigella spp* vaccine is compatibility for combination with other enteric vaccines to be given by the same route.

OBJECTIVE

- Isolation of *shigella spp* from infected stool sample.
- Preparation of antigens of *shigella spp*.
- To check purity and sterility of the prepared antigen.
- To check specificity of the prepared antigen using ELISA

2. MATERIALS AND METHODS

3. 3.1: COLLECTION OF SAMPLES

Fig.1 Stool sample of infected patient.

Stool samples were collected from PSG IMSR Coimbatore, this sample was used for this study. This sample was pure cultured on nutrient broth and was forwarded for further confirmed by staining and culture methods.

3.2 : ISOLATION OF THE ORGANISMS FROM PURE-CULTURES

3.2.1 : Gram's staining

Dr. Hans Christian Gram, a Danish physician, developed the Gram Stain in 1884. It is very useful to identify and classify bacteria into two major groups such as Gram-positive and Gram-negative. By this method to perform identify the bacterial group.

3.2.2 Biochemical characterization

Catalase test

Accumulation of hydrogen peroxide and super oxide leads to the death of the organisms unless they are degraded enzymatically, organisms capable of producing catalase or peroxidase. 1ml of 3% hydrogen peroxide was added to the culture after 5 minutes the culture was examined immediately for the evolution of bubbles, which indicates a positive test.

Indole test

Tryptophan is decomposed in to its metabolic products like indole, pyruvic acid and ammonia by the enzymes, tryptophanase. The indole is detected by calorimetric reaction by p – dimethyl amino benzaldehyde (kovac 's reagent). The indole was performed and obtained the result.

Oxidase test

During aerobic respiration, oxidase enzymes play a vital role in the operation of electron transport system. Cytochrome oxidase the catalyzes the oxidation of a reduced cytochrome by molecular oxygen, resulting in the formation of water or hydrogen peroxide. This test depends on the presence of certain oxidases in bacteria that will catalyzes the transport of electrons between electron donors in the bacteria and a redox dye – Tetramethyl p-Para phenylene diamine dihydrochloride. The dye is reduced to a deep purple color.

Citrate utilization

During this reaction, the medium becomes alkaline as the carbon dioxide combines with sodium and water to form sodium carbonate changes the bromothymol blue from green to deep Prussian blue. A positive tube shows a blue color on the streak of growth. Retention of original green colour and no growth on the line of streak indicates a negative reaction.

Methyl red test

A loopful of bacteria was inoculated in MRVP broth. It was incubated for a time periodof 3-5days. After incubation the result was analyzed from the broth. A clear broth indicates that the organism did not grow and cannot be tested; 1ml of broth was removed and placed into a sterile tube before performing the methyl red test 3-4 drops of methyl red was added to the original broth. A positive result has a distinct red layer at the top of the broth. A negative result has a yellow layer.

Voges-proskauer test

The VP A (naphthol) reagent and 5 drops of the VP B reagent (potassium hydroxide) was added. This reaction takes few minutes before a colour change. With a positive reaction the medium changes to pink or red. While a negative reaction the broth will not change colour orwill be copper colored.

Triple sugar iron agar test

The TSI agar medium was prepared, sterilized and poured into sterile tubes. Using a sterile inoculation needle, the slants were inoculated by first stabbing the butt down to the bottom and then streaking the surface of slant. Following inoculated at 37 ˚C for 24 hours.

Urease test

The test cultures were inoculated heavily over the entire slope culture of Christenson's agar

medium and incubated at 37^0 C for 24 hours and the reactions are recorded after 48 hours of incubation. A positive urease reaction is indicated by a change in the colour of the medium from yellow to purple colour, negative urease reaction shows no colour change of the medium.

3.2.3. CULTURE PLATES AND SELECTIVE MEDIA

Nutrient agar

Nutrient agar is a common medium used for the culturing of all types of organisms. It is basal medium which supplies the basic requirements of carbon, nitrogen and mineral source for growth. Distilled water was taken in a conical flask and the chemicals were dissolved in to it. Nutrient agar was prepared and culture were inoculated.

Xylose lysine deoxycholate (XLD)

This agar is a selective medium for the isolation of *shigella* and *salmonella spp* from clinical specimens and food samples. XLD Agar was originally formulated for the isolation and identification of *shigella* from stool sample. This medium was formulated to increase the frequency of growth of the more fastidious pathogens, which in other formulation have often failed to grow due to the inclusion of excessively toxic inhibitors. After solidification of the medium, using a sterile loop, the culture was quadrant streaked on to the XLD agar plates.

Salmonella shigella agar (SS)

SS Agar is moderately selective and differential medium for the isolation, cultivation and differentiation of *salmonella spp*. and some strains of *shigella spp.* SS Agar is the modification of the Deoxycholate Citrate Agar. It is recommended for testing clinical specimens and food testing for the presence of *salmonella spp*. and some *shigella spp*. Distilled water was taken in a conical flask and the chemicals were dissolved in to it. After cooling, the medium was poured in to sterile petri dishes. After solidification of the medium, using a sterile loop, the culture was quadrant streaked on to the SS agar plates.

3.3 : ANTIGEN PREPARATIONS

Distilled water was taken in 4 conical flask and the chemicals were dissolved in to it. PH was dissolved to 7.2-7.4.the medium was sterilized at 121℃ for 15minutes in an autoclave. After cooling, the nutrient broth was inoculated with 4 strains of *shigella spp* culture, using a sterile loop and incubated at 37° C for 48hours.

Centrifuge the *shigella spp* cultures in 4 tubes. At 7000rpm for 15mins.Discarded the supernatant. Collect the pellets and suspended with the PBS buffer (pH: 7.4). Centrifuge again and discard the supernatant. Wash the pellets with PBS buffer (Three times). Final pellets were collected and stored for the further process.The pellets were was exposed to heat by heating for 30 mins 100℃ or add 5% formalin and left for 48hrs at room temperature for killing of antibody.

3.3.1: STERILITY TESTING

A loopful of the killed antibody was inoculated to the selective medium and was incubated for 24hrs at 37℃ and was observed.

3.3.1 : PURITY CHECKING

The purity was checked by gram' staining method and viewed under microscope.

3.4 : ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

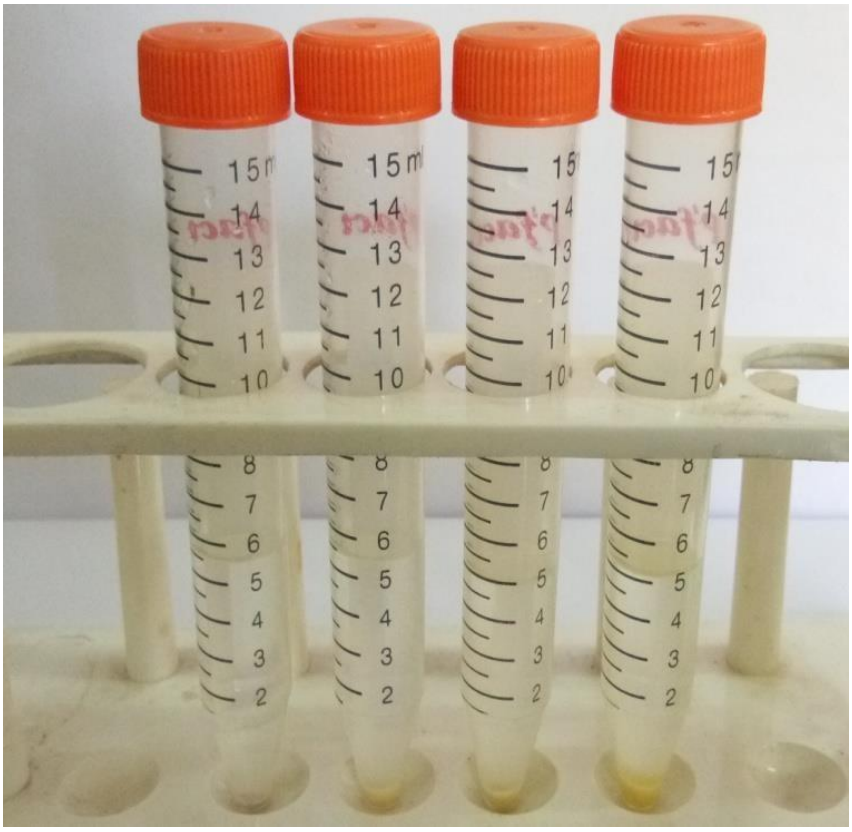
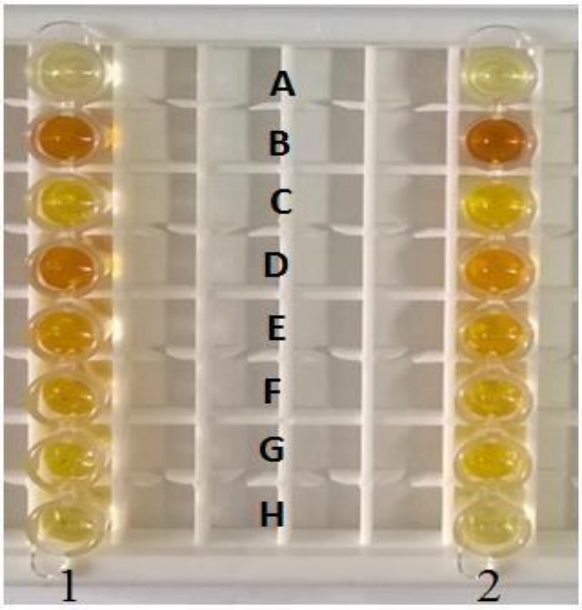


Fig.2 Formalin killed antigen (*Shigella spp*) samples.

3.4.1. ELISA TESTING (INDIRECT ELISA)



A – Blank
B – Neat (Positive Control)
C – Negative Control
D – 1:10
E – 1:100 Dilution
F – 1:1000 Dilution
G – 1:10000 Dilution
H – 1:100000 Dilution

Fig: 3: 1: Anti-*Shigella sonnei* IgY Extract, 2: Anti-*Shigella boyaii IgY* Extract.

A – Blank
B – Neat (Positive Control)
C – Negative Control
D – 1:10
E – 1:100 Dilution
F – 1:1000 Dilution
G – 1:10000 Dilution
H – 1:100000 Dilution

Fig.4: 3: Anti-*Shigella flexneri* IgY Extract, 4: Anti-*Shigella dysenteria* IgY Extract.

Determination of antibody titer by Indirect ELISA

The immunological specificity of IgY elaborated against ***Shigella sonnei, Shigella boyaii, Shigella flexnari*** and ***Shigella dysenteria*** antigens were examined by enzyme-linked immunosorbent assay (ELISA). In brief, wells of Microtiter plates were coated with 100 µl of antigen solution (A660nm=1.0) appropriately diluted with 0.05 M carbonate buffer (pH 9.6). After overnight incubation at 4°C, the plates were washed, and 200µ1 of PBS (pH 7.4) containing bovine serum albumin (1% in PBS) was added to the wells in order to block the uncoated surface. After being blocked each well was washed three times with 200 µl of PBS

(0.85% NaCl-0.01 M phosphate buffer, pH 7.2)-Tween (containing 0.05% Tween 20), and IgY from immunized hens at different time intervals was applied to the well in triplicate for reaction with their respective antigen for 2 hours at 37 °C. After each well was washed again with 200µL of PBS-Tween, 100µL of horseradish peroxidase-conjugated rabbit anti-chicken IgG (Sigma Chemical Co.) diluted (1:1000) with PBS-Tween was added to each well, and the plate was incubated at 37 °C for 2 hours. Each well was washed again with 200 µl of PBS-Tween and then 100 µl of TMB solution with H_2O_2 (GeneiPvt. Ltd., Bangalore). The reaction was stopped after 20 min with 4N H_2SO_4 (50µl per well), and the intensity of color developed was measured at 490 nm with a micro-plate reader. Crude non-specific IgY from non-immunized hens were used as control. All samples were tested in triplicates.

4. RESULT AND DISCUSSION

4.1 : GRAM'S STAINING

After staining the result was **gram negative rod shape.** Those that lose the crystal violetand counter stained by safranin (appear red) are referred to as Gram-negative.

4.2 : BIOCHEMICAL CHARACTERIZATION

Table.1: Biochemical test.

Organisms	Glucose	Sucrose	Mannitol	Lactose	TSI	Indole	MR	VP	Citrate	Urease	Catalase	Oxidase
Shigella spp	+	-	+	-	AK/A No gas	+	+	-	-	-	+	-

4.3 : CULTURE PLATES

- **NUTRIENT AGAR**

After incubation transparent colourless colonies with rough appearance was observed.

- **XYLOSE LYSINE DEOXYCHOLATE (XLD)**

After incubation *shigella* is red oily, rough irregular colonies were observed.

- **SALMONELLA SHIGELLA AGAR (SS)**

After incubation *shigella* are smooth and opaque or colourless was observed.

5. CONCLUSION

The present study describes a means of generating *Shigella antigen* that elicits high titers of specific polyclonal antibody. The anti-*Shigella* antibodies generated can detect protein antigen. For the detection of *Shigella* in samples, optimization of the technique would be required. Samples could be cleaned up prior to indirect-ELISA, or immune analysis could be

performed. Although there were few medical complications associated with shigellosis, control of this disease could reduce of the overall diarrhea burden globally. The development of a vaccine protective against shigellosis is a highly desirable public health goal, but the development of such a vaccine is complicated by the variation in species and sero-groups between sites, years, and age groups. The food and water borne pathogen *Shigella* has huge public health importance and the emergence of antibiotic resistance in them demands the development of new and better antimicrobial drugs. However, there is possibility that they might evolve and become resistant to those newly formulated drugs.

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