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European Journal of Experimental Biology, 2015, 5(7):49-61

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Recovery of *Salmonella* and *Shigella* isolates from drinking water

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ABSTRACT

The occurrence and survival of enteric bacteria in treated potable waters has been of great concern to health and well being of society. Present study reported the prevalence of Enteric Bacteria; Salmonella and Shigella in disinfected effluent entering the distribution system and which finally serving the end-users. Salmonella and Shigella were detected and quantified by Direct Plating Method from a total of seventy two samples from public water supply of Neemuch, Madhya Pradesh. Isolates were further characterized morphologically, culturally and biochemically. In raw influent, both the enteric groups showed presence in dense amounts in all the samples. Although treatment practices at the plant significantly reduced their concentrations (P<0.05), yet their numbers rose unexpectedly within the distribution system, consequently water distributed to consumers contained Salmonella and Shigella in concentrations nearly similar to those in raw influent water. Furthermore, no tested sample from the treatment plant and the points of consumption met recommended WHO and BIS health standards (zero pathogen/ml) (P<0.05), which presents potential health hazard and would place consumers at risk.

Key words: Disease, Effluent, Health, Treatment

INTRODUCTION

The majority of infections associated with drinking water are those which cause gastro-enteritis. Almost all enteric pathogens that are transmissible by the fecal-oral route can be transmitted through water. However, the rate of inactivation in the water environment and infectious dose are the critical characteristics of an organism that defines the risk of a waterborne outbreak of disease. *Vibrio cholerae, Shigella spp., Salmonella spp., Campylobacter, Giardia lamblia,* and *Cryptosporidium parvum* can clearly be considered waterborne pathogens (other routes of infection are food, soil, person to person, etc.); however, they are all enteric pathogens that may survive but cannot proliferate in treated drinking water.

Salmonella is a ubiquitous intestinal pathogen with a worldwide distribution that comprises a large number of serovars characterized by different host specificity and distribution. This organism is one of the leading causes of intestinal illness all over the world as well as the etiological agent of more severe systemic diseases such as typhoid and paratyphoid fevers [1]. *Shigella* is typically an inhabitant of the intestinal tract of humans and other primates and is excreted in large numbers in the feces of infected individuals. It is typically spread by fecal-contaminated drinking water or food or by direct contact with an infected person. Its presence in the population is maintained by a few symptomless carriers. In water, shigellae can survive for at least six months at room temperature, and this high

survival favors transmission through water. The total number of *Shigella* episodes that occur each year throughout the world is estimated to be 164.7 million, including 163.2 million cases in developing countries, 1.1 million of which result in death. Children under 5 account for 61% of all deaths attributable to shigellosis [2,3]. In many developing countries with inadequate sanitation, fecal contaminations of environmental waters by enteric pathogens are very common and natural waters are major source of microbial pathogens. These water bodies ultimately serve as municipal raw supplies, which indicate possibilities for the transmission of these pathogens to the end-point users. The present paper presents an investigation on occurrence, enumeration, seasonal incidence and characterization of enteric bacteria; *Salmonella* and *Shigella*, recovered from *Jaju Sagar Dam* Neemuch, related purification plant and point of use.

MATERIALS AND METHODS

Sampling Sites

Inlet of the plant was sampled for the collection of raw influent and two sites were chosen from the chlorinated supply for the testing of potable water; *Outlet* of treatment plant and *Point of consumption*.

Sample Collection

Water samples were collected every fortnight from each site for one complete year. Thus, 24 samples were collected per site from January–December 2013. Water samples were collected into sterile bottles in 100 ml amounts and processed within 2 hours of collection. Heat sterilized bottles containing a sufficient volume of sodium thiosulphate (0.1 ml of 1.8 g/100 ml sample) to neutralize the bactericidal effect of residual chlorine was used to collect water from the chlorinated supply. The mouths of taps were flamed to prevent environmental contamination. After collection, the bottles were stoppered and labeled with full details concerning source, time and date of collection. The samples were kept in coolers packed with ice and transported to the laboratory within 2 hours and protected from light.

Sample Analysis

Primary isolation of *Salmonella* and *Shigella*: Salmonella Shigella agar which is a selective and differential medium for the isolation of enteric pathogens was used for the isolation and enumeration of *Salmonella* and *Shigella* by means of Direct Plating Method.

Identification of presumptive isolates: Colonies of presumptive *Salmonella* spp. (colourless & black centered) and *Shigella* spp. (colourless) were counted after confirming their identity through some biochemical tests; Triple sugar iron agar test, Urease production test, Indole production test and Motility test.

Characterization of isolates: Morphological characteristics of *Salmonella* and *Shigella* were studied by using conventional microbiological techniques viz. light microscopic observations of gram-stained smears. Motility was tested by stabbing the culture into deep tubes of mannitol motility test medium (Himedia, Mumbai, Maharashtra, India), appearance of cloudiness was evident for motility. To study cultural characteristics, colony morphology was observed on Salmonella-Shigella agar as well as on nutrient agar. Type of growth on nutrient agar slant and in nutrient broth was also studied. Oxygen requirement of the isolates was determined by inoculation in fluid thioglycollate medium (Himedia, Mumbai, Maharashtra, India) deep tubes. Biochemical characterization of the isolates was done by performing some additional biochemical tests (Table: 3).

RESULTS

Occurrence and Enumeration of Isolates

Table 1 presents fortnight log transformed counts of *Salmonella* and *Shigella* in raw influent and disinfected effluent samples. Figures 1 (*Salmonella*) and 2 (*Shigella*) illustrate monthly trend in the counts from the three kinds of supplies. Results of present study revealed that *Salmonella* and *Shigella* had 100% frequency of occurrence in inlet water samples. During all the sampling months minimum *Salmonella* count (1.75) in raw influent samples was obtained in April (S2) and maximum (2.23) in May (S1) whereas *Shigella* counts fluctuated between1.84 (Feb S1) to 2.27 (July S1). Seasonally, a common trend was observed in the counts of both the groups; rainy>summer>winter. Seasons were categorized as summer: March-June, rainy: July-October, and winter: November to February. Seasonal average log count of *Salmonella* in winter season was 1.97 while showed closest values in summer and

rainy seasons- 2.03 and 2.07 respectively. The seasonal average log count of *Shigella* in winter was 1.97, in rainy 2.20 whereas in summer it was 2.10 (fig 3). Conventional treatment methods (coagulation, filtration and disinfection) at the plant significantly affected microbial populations; the counts reduced temporarily as a result the frequency of isolation were slightly lower. *Salmonella* occurred in 87.5% effluent water samples ranging between 0.51 (Apr S2 & Nov S1) and 1.69 (June S2), and *Shigella* was present in 91.6% varying between 0.51 (Oct S1) and 1.82 (March S2) (table 1). During each month of the sampling year, disinfected effluent samples from the outlet were having decreased amounts of counts than the raw influent samples ($P<0.05$), as expected, yet the counts remained higher than the defined detection limit of WHO [4] and BIS [5] ($P<0.05$). According to the recommended drinking water standards, there is no tolerable lower limit for pathogens in water intended for consumption, preparing food, drink or for personal hygiene; it should contain no bacteria pathogenic to humans. But in contrast to this, throughout the whole study period, no sample from the outlet showed complete absence of both the pathogens; 79.1% samples had presence of both *Salmonella* and *Shigella* whereas 12.5% samples were contaminated with *Shigella* only and 8.4% samples harboured *Salmonella* only. As it is well documented that, the water that reaches to the consumer's tap is often of worse quality than that left the plant. Unfortunately, in present study also it was found that the counts significantly increased within the distribution system up to the final destination resulting into 100% frequency of isolation at the point of use. Final water consumed by the public had densities of enteric bacteria above the health standards ($P<0.05$); *Salmonella* counts ranged from 1.60 (Oct S1) to 2.14 (July S2) and *Shigella* counts from 1.30 (Feb S2) to 2.19 (March S1). This shows that the final water in the tap was also not in accordance with the recommended standards.

Table 1: *Salmonella* and *Shigella* densities in drinking water samples

Months		Raw Influent		Disinfected Effluent			
				Outlet		Point of Use	
		Salmonella Log counts/ml	*Shigella* Log counts/ml	*Salmonella* Log counts/ml	*Shigella* Log counts/ml	*Salmonella* Log counts/ml	*Shigella* Log counts/ml
January	S1	1.86	1.90	1.00	1.63	1.75	2.07
	S2	1.77	1.96	1.42	1.72	1.90	1.86
February	S1	1.90	1.84	0.00	1.30	1.84	1.90
	S2	2.00	1.86	1.60	0.00	1.96	1.30
March	S1	2.02	2.11	1.22	1.47	1.80	2.19
	S2	2.06	1.93	1.30	1.82	2.00	1.92
April	S1	1.95	2.23	1.00	1.30	1.72	2.08
	S2	1.75	2.04	0.51	1.00	2.04	1.82
May	S1	2.23	2.19	1.12	1.69	1.84	2.13
	S2	2.15	1.95	1.56	1.60	2.02	2.01
June	S1	2.13	2.21	1.47	1.36	2.05	1.90
	S2	1.96	2.11	1.69	1.60	1.72	2.07
July	S1	2.19	2.27	1.41	1.72	2.01	2.02
	S2	2.14	2.23	1.52	1.80	2.14	2.13
August	S1	2.11	2.25	1.60	1.60	1.91	1.93
	S2	2.10	2.17	0.00	1.00	2.11	2.05
September	S1	2.07	2.26	1.30	1.42	1.84	1.86
	S2	2.02	2.15	1.00	1.75	2.09	2.02
October	S1	1.90	2.08	1.63	0.51	1.60	2.06
	S2	2.05	2.16	0.00	1.69	1.75	1.77
November	S1	1.95	2.00	0.51	1.56	1.82	1.63
	S2	2.11	2.17	1.42	1.30	1.90	2.16
December	S1	2.14	2.06	1.66	0.00	1.95	1.47
	S2	2.01	1.98	1.47	1.52	1.69	1.75
Frequency of occurrence		100%	100%	87.5%	91.6%	100%	100%
Range		1.75-2.23	1.84-2.27	0.51-1.69	0.51-1.82	1.60-2.14	1.30-2.19

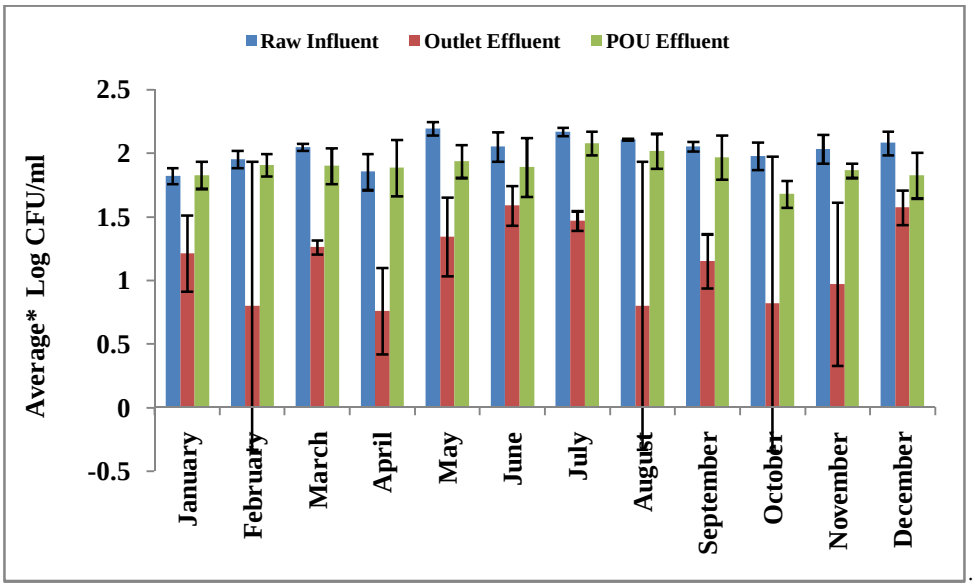


Fig 1: Fluctuations in S*almonella* densities
**Mean of S1 & S2 readings*

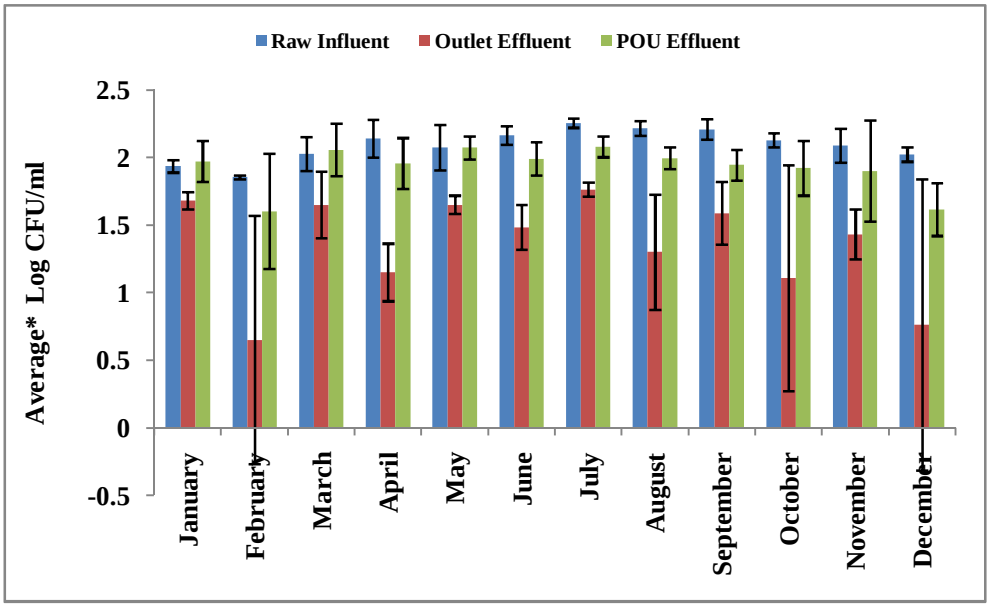


Fig 2: Fluctuations in *Shigella* densities
**Mean of S1 & S2 readings*

Characteristics of Isolates

Gram staining of *Salmonella* isolates showed straight, gram negative, small rods, singly and in pairs and *Shigella* exhibited gram negative short rods but mostly singly arranged. Appearance of cloudiness and thin spreading filaments in mannitol motility test medium inoculated with *Salmonella* culture confirmed the motility of the cells whereas all the isolates of *Shigella* were found to be non-motile due to the lack of spreading filaments. Isolates of both the groups were non-sporeforming and non-capsulated.

Overnight growth (18 to 24 hours) of *Salmonella* on Salmonella-Shigella agar produced large (2 to 3 mm in diameter), slightly raised, black centered colourless colonies with entire margin (Table 2). The black center was actually an insoluble black precipitate of ferrous sulphide, formed upon reaction of H_2S with ferric ions or ferric citrate, which indicates the production of H_2S by *Salmonella*. *Shigella* produced small, round, colourless colonies with entire margin and convex elevation. On nutrient agar plates colonies of *Salmonella* and *Shigella* isolates were white, opaque, round with entire margin. Isolates showed filiform type of growth on nutrient agar slant and in nutrient broth mostly *Salmonella* produced pellicle and *Shigella* showed membranous type of surface growth. Turbid type of sub-surface and flocculent type of sediment growth was shown by most of the isolates of the two groups. Results of biochemical characterization are presented in table: 3.

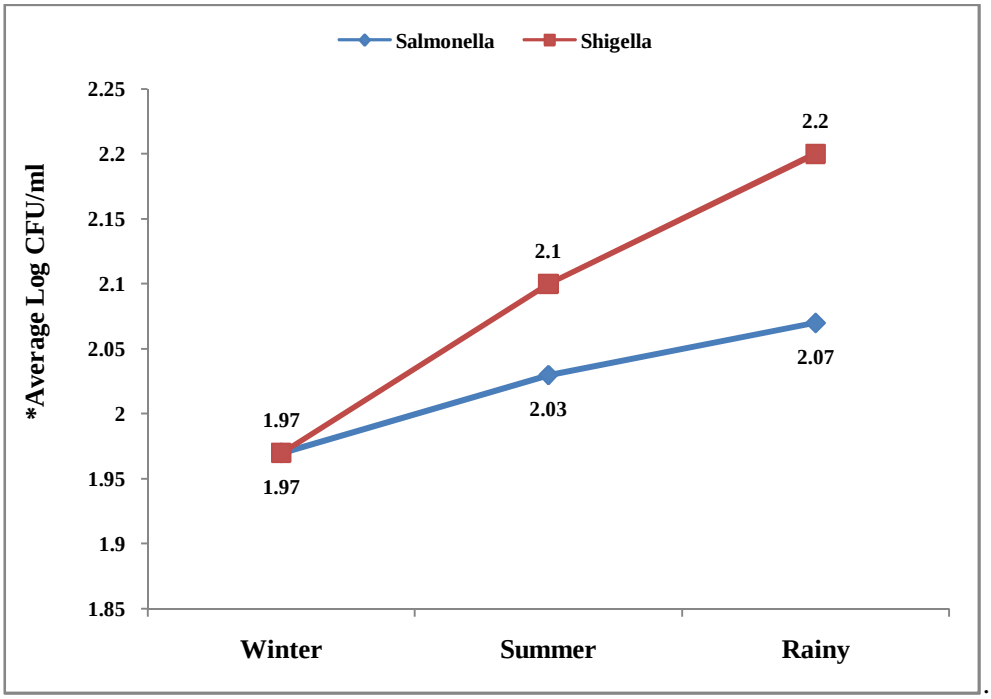


Fig 3: Seasonal variability in *Salmonella* and *Shigella* counts
**Mean of four months*

Table 2: Cultural Characteristics of the isolates

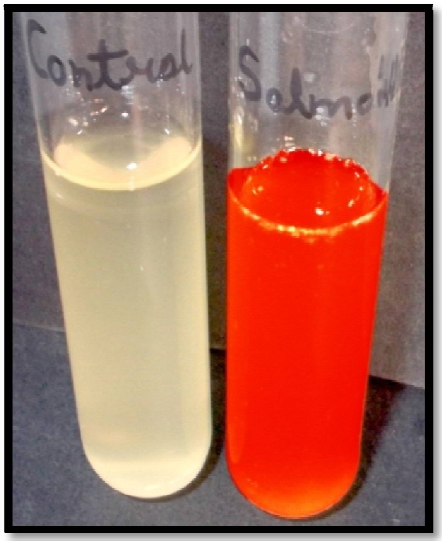
Cultural Characteristics	*Salmonella*	*Shigella*
Type of growth on NA slant-		
Amount of growth	Thin	Thin
Color	Grayish	Grayish
Opacity	Opaque	Opaque
Form	Filiform	Filiform
Oxygen requirement	Facultative Anaerobic	Facultative Anaerobic
Colony on NA plate-		
Form	Round	Round
Color	White	White
Margin	Entire	Entire
Elevation	Raised	Convex
Growth in nutrient broth-		
Amount of growth	Slight	Abundant
Surface growth	Pellicle	Membranous
Sub-surface growth	Turbid	Turbid
Sediment growth	Flocculent	Flocculent
Colony on Selective Media-		
Form	Round with raised margin	Round
Color	Black centered colourless	colourless
Margin	Entire	Entire
Elevation	Raised	Convex & Flat

Table 3: Physiological Characteristics of the isolates

Biochemical Characteristics	*Salmonella*	*Shigella*
Indole Test	-	+
Methyl Red (MR) Test	+	+
Voges- Proskauer (VP) Test	-	-
Simmon Citrate (SC) Test	+	-
Oxidase Test	-	-
Catalase Test	+	+
Oxidative-Fermentative (OF) Test	F	F
Nitrate Reduction (NR) Test	+	+
Mannitol Fermentation Test	+	+
Amylase Test	-	-
Gelatinase Test	-	-
Urease Test	-	-
TSIA- Glucose	+	+
Gas from Glucose	+	-
Sucrose	-	-
Lactose	-	-
H_2S	+	-

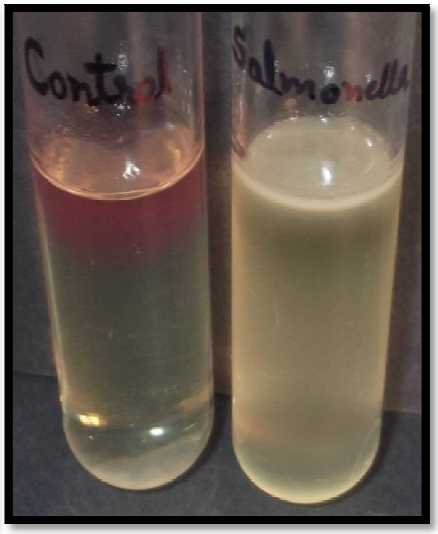
+ = 90 to 100% of the isolates were positive;
- = 0 to 10% of the isolates were positive

Negative Indole Test



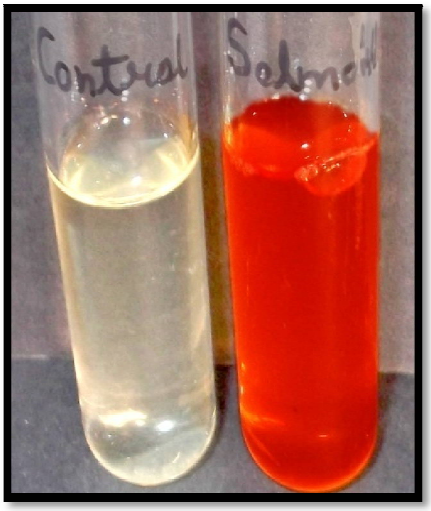
Positive MR Test

Negative VP Test



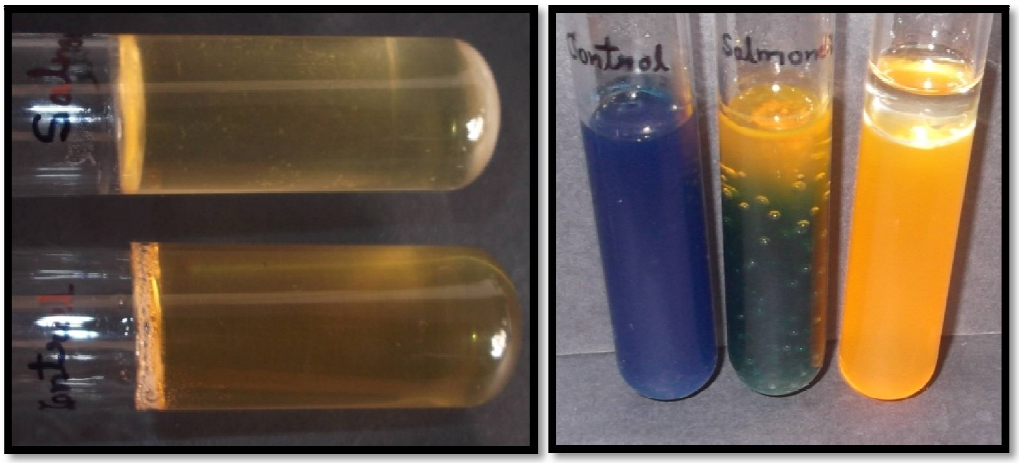
FTG Test – Facultative Anaerobic

Negative Urease Test

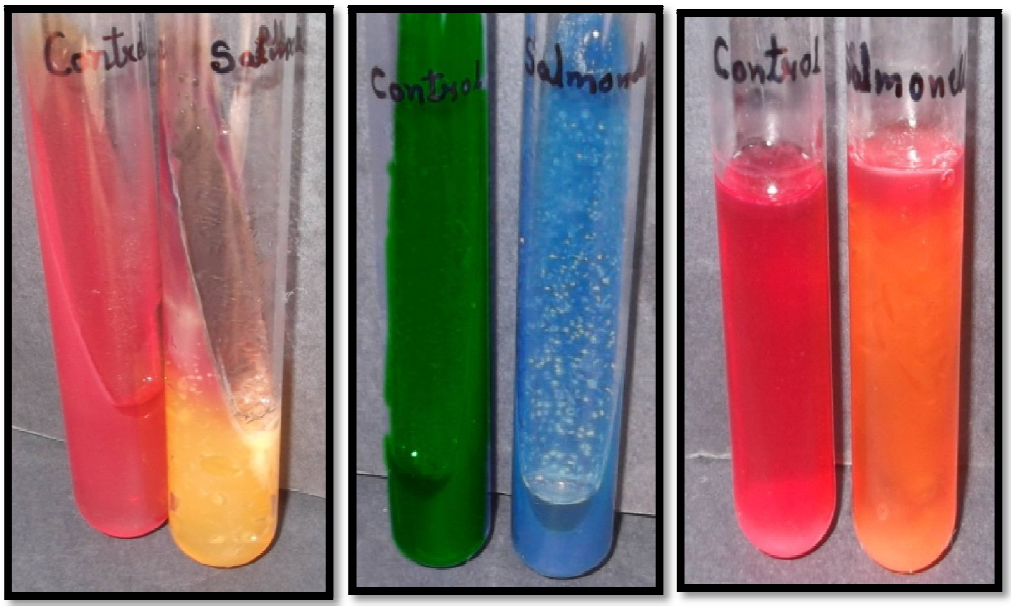


Positive NR Test

Negative Starch Test **Negative Oxidase Test** **Positive Catalase Test**

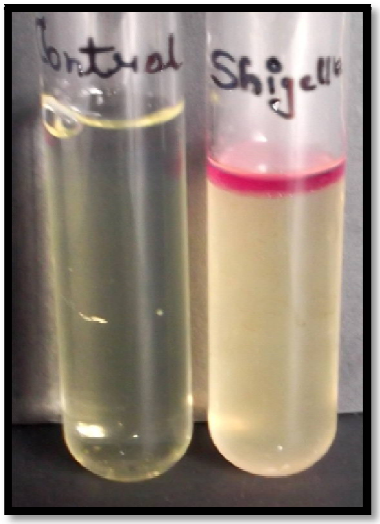


Negative Gelatinase Test **OF Test - Fermentative**



Positive TSIA Test **Positive SC Test** **Positive Mannitol Test**

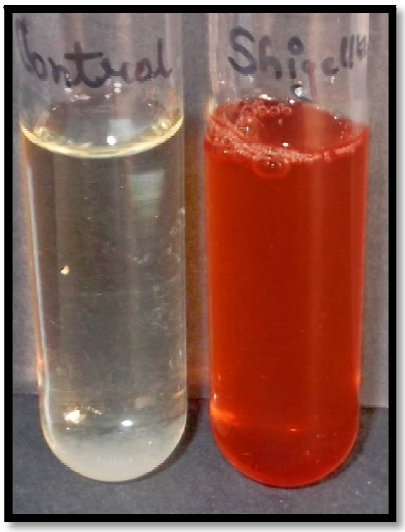
Fig 4: Biochemical results of *Salmonella* spp.



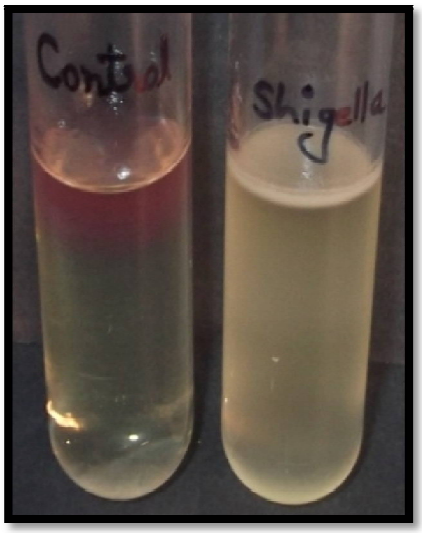
Positive Indole Test

Positive MR Test

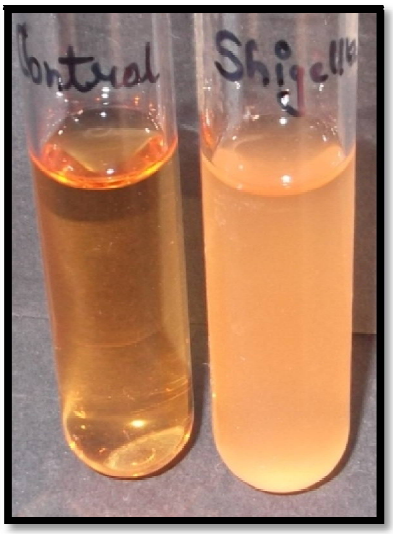
Negatve VP Test



Positive NR Test



FTG Test - Facultative Anaerobic



Negative Urease Test

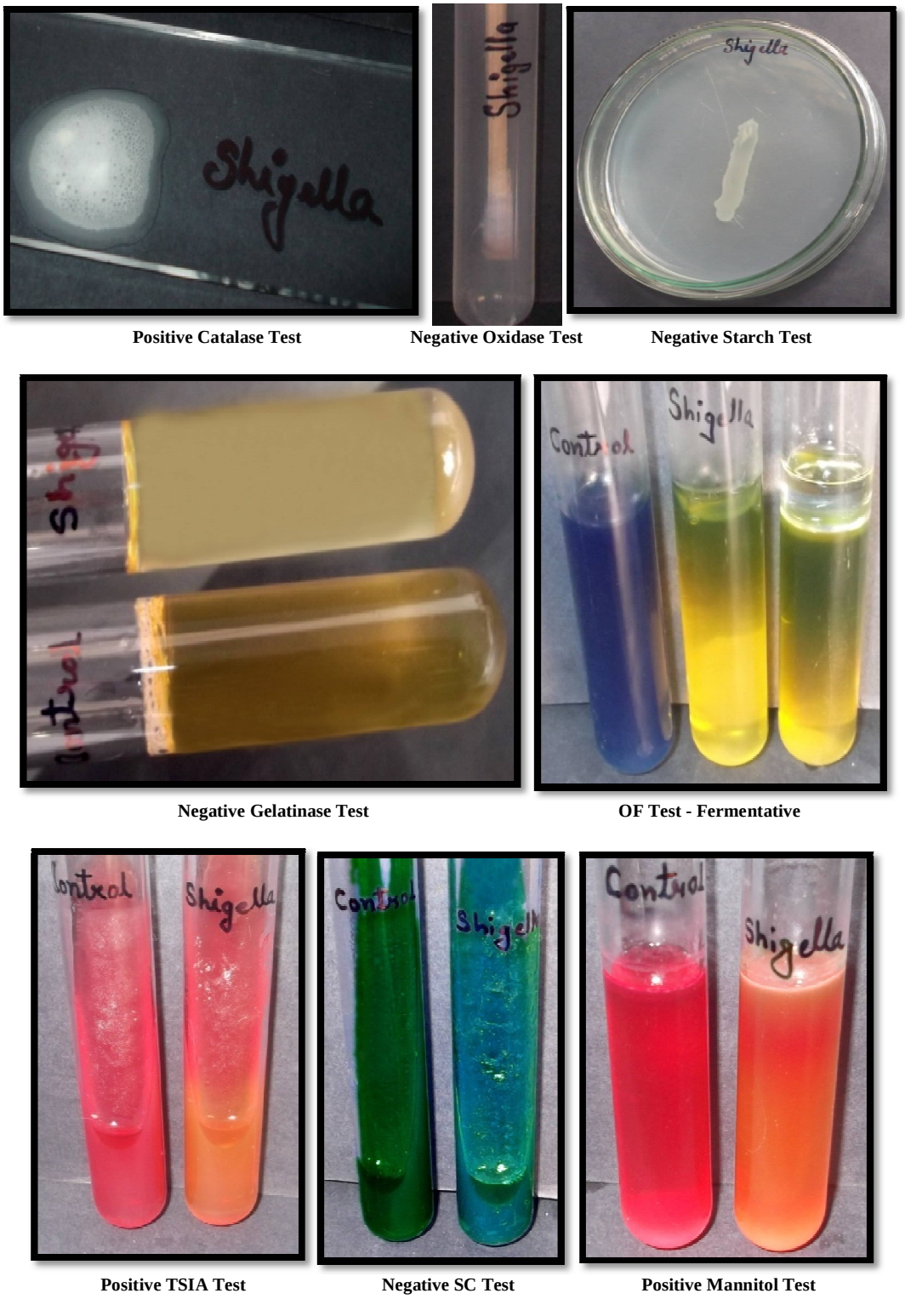


Fig 5: Biochemical results of *Shigella* spp.

DISCUSSION

The principal habitat of *Salmonella* is the intestinal tract of humans and animals, and *Shigella* is human specific. These are constantly found in environmental samples, because they are excreted by humans, pets, farm animals, and wild life. In the aquatic environment, these pathogens have been repeatedly detected in various types of natural waters such as rivers, lakes, coastal waters, estuarine as well as contaminated ground water. The direct sources of these pathogens in natural waters are fecal matter from the infected people and indirect are the household sewage discharge, municipal sewage, agriculture pollution, storm water runoff etc. After entering the natural environment, these enteric bacteria do not seem to multiply significantly, but they can survive several weeks in water and in soil if conditions of temperature, humidity, and pH are favorable. Incidences of Salmonellosis and shigellosis outbreaks as a result of poor water quality have been reported throughout the world. The results of present study are consistent with many of the previous studies [6,7,8,9] which reported the usual occurrence of these enteric bacteria in natural raw waters. Present results slightly vary to that of Osman et al. [10] who detected salmonellae group in Nile raw water, Egypt whereas all the outlet and tap water samples, tested by him were free from salmonellae groups. In agreement to the current findings Ahmed et al. [11] also recovered *Salmonella* and *Shigella* spp. from water samples of different dams and related filtration plants of Rawalpindi, Islamabad region in Pakistan. Likewise, Yongsi et al. [12] identified 1,242 isolates of enteric bacteria from a variety of drinking water sources of Yaounde, of which *Shigella* had 0.24% and *Salmonella* had 1.30% occurrence. Ihejirika et al. [13] also recovered *Shigella* spp. (71.0%) and *Salmonella* spp. (71.0%) from Imo River, Nigeria. Sila et al. [14] isolated *Shigella* spp. from the Lamingo Dam Jos Nigeria, its water filter tanks and water taps. Obi et al. [15] obtained *Shigella* and *Salmonella,* in water sources of the Venda Region of the Limpopo Province, with a higher tendency in the months of summer in comparison to other seasons ($p<0.05$). Eleven different kinds of enteric bacteria were isolated by Jayana et al. [16] from different drinking water sources of Madhyapur Thimi which included *S.dysentery* (2.8%), *S.typhi* (2.1%) and *S.paratyphi* (1.4%). Jafari et al. [17] reported different serotypes of *Salmonella* from drinking water in Broiler Farms in Iran. Similar to our finding, Haley et al. [18] in his study on ecology of *Salmonella* spp. in a Southeastern watershed, Georgia, also obtained highest *Salmonella* densities in summer months and lowest in winter months. Egwari and Aboaba [19] isolated *Salmonella* and *Shigella* from domestic water supplies in Lagos, Nigeria. Kinge et al. [20] investigated the occurrence and distribution of *Shigella* species in river catchments in the North West province of South Africa. *Shigella sonnei* was isolated by Lindell et al. [21] from well water samples in Iowa. Hatha et al. [22] observed increased prevalence of *Salmonella* (42-57%) in Vembanadu Lake, along west coast of India. The findings of this and previous studies confirm the prevalence of enteric pathogens in surface raw waters and indicate that surface waters can possibly transport these bacteria, and there is a possibility of a continuous source of contamination into the aquatic bodies. The detection of the intestinal pathogens in the raw influent samples in present study suggests the likelihood of other pathogenic bacteria and confirms the presence of fecal contamination in *Jaju Sagar Dam*. The observed monthly and seasonal variability in the counts may be due to the fluctuations in the availability of required physical and other hydrological characteristics of dam water, which greatly affects the growth and survival of the pathogens. The most important ones are temperature and pH of water. Higher prevalence in summer indicate that being human pathogens both the groups of bacteria met the required warmer temperature of water during summer months close to that of human body. Rainfall is also an important factor which may be attributable for deteriorating the dam water quality. Samples collected after heavy rainfall contained highest density of counts, probably due to rainfall runoff from agricultural lands which provided a mechanism for the transfer of fecal wastes to *Jaju Sagar Dam.*

Water for human consumption is usually disinfected before being distributed to the consumer to ensure that the level of any potentially harmful microbial agent falls under defined low levels. In many instances the quality of the water may have deteriorated by the time it reaches to the consumer. This is often due to recontamination after treatment owing to the regrowth of sub-lethally damaged bacteria or contamination from bacteria harboured in biofilms. In present study also, the numbers of both the enteric groups decreased temporarily after treatment and chlorination, but further increased within the distribution system. The results point out inefficient working of purification plants in developing countries to produce water of an acceptable quality and to prevent post-treatment changes as well, hence, the results pose great risk on health status of those inhabitants who rely solely on municipal water supplies. Securing the microbial safety of drinking-water supplies is based on the use of multiple barriers, from catchment to consumer, to prevent the contamination of drinking water or to reduce contamination to levels not injurious to health. Safety is increased if multiple barriers are in place, including protection of water resources, proper selection and operation of a series of treatment steps and management of distribution systems (piped or otherwise) to maintain and protect

treated water quality. The use of a multiple-barrier approach can reduce these pathogens to non-detectable levels or to levels that have never been associated with human illness. The preferred strategy is a management approach that places the primary emphasis on preventing or reducing the entry of pathogens into water sources and reducing reliance on treatment processes for removal of pathogens. As no treatment method can be effective alone and no treatment plant can produce a sterile product, hence, to ensure delivery of safe water at the consumer's tap, protection of source water is the most important. Aquatic environments receive a significant number of human microbial pathogens from point and non-point sources of pollution. The water quality in *Jaju Sagar Dam* has been deteriorated due to the frequent contamination by diverse group of pollutants. This dam water is the actual source for the origin of enteric pathogens in finished water, hence it is strongly suggested that *Jaju Sagar Dam* must be protected from any kind of fecal contamination to avoid possible diseases outbreak and transmission. In addition, a clean environment established through provision of adequate infrastructure for disposal of refuse and feces will reduce the level and frequency of contamination of water supply systems.

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