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GENETIC DIVERSITY AMONG *SALMONELLA* SPECIES ISOLATED FROM SOIL AND SEWAGE SAMPLES IN NANDED, INDIA

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ABSTRACT

A total of fourty four *salmonella* spp. isolates randomly from sewage and soil samples were included for the genetic closeness and diversity study using Pulsed Field Gel Electrophoresis (PFGE) and Bionumerics. X*bal* restriction enzyme band pattern was produced and the same was analyzed using Applied Maths software. Standard ISO method was used for the isolation of the bacteria from the samples. The suspect isolates were confirmed using RT PCR technique for fast processing and reliability of the result. 24-27 hours standard protocol for *salmonella* spp. from World Health Organization (WHO) was used for the PFGE run setup. All 44 isolates produced clear finger print pattern and out of which 33 were grouped in to one cluster (Cluster A) and the rest 13 into Cluster B. The cluster A contains 4 groups which exhibited 100% similarity in their genetic structure, out of which the group one and two contains 6 and 3 isolates, while group three and four contained 2 isolates each. Cluster B had only two groups with 100% similarity and the first group with 8 and the later with 2 isolates. There were 18 isolates which was not genetically similar to each other in cluster A, and 3 in cluster B. From the result it was able to conclude the spread of *salmonella* spp. in sewage and soil across various area of Nanded district.

KEY WORDS: **Genetic diversity, Pulsed Field Gel Electrophoresis, *Salmonella species,* X*bal* digestion, Molecular Typing**

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INTRODUCTION

Salmonella is one of the most widely studied bacteria that cause salmonellosis and they are a major concern in the developing countries[1, 4, 21]. *Salmonella* spp. are gram negative with flagellated structure, and they are non-spore forming and survive as facultative anaerobic bacteria. They grow at an optimum temperature range between 35 and 37^{0}C [1]**.** The sources of these bacteria are mostly from the avian and mammalian species and they grow in the intestinal tract and largely associated with poultry[15]. Infections caused by *salmonella* spp. results in more than 600 deaths annually in U. S alone, and many more worldwide, especially in developing countries. The money spend on treatment and post production losses are estimated over billion dollars annually (U.S FDA, 1996). In the modern time the international trade among countries, travel, globalization and other activities expedite and enable the transmission of such food borne pathogens [4]. There are about 21 million cases reported worldwide with typhoid fever annually, and the majority of this reported in Asian countries. Close to 220,000 deaths are registered due to *salmonella* infections in Asia[9]. World Health Organization (WHO) has reported more than 2600 of *salmonella* strains (serovars or serotypes) with two main subspecies, *Salmonella enterica* and *Salmonella bongori*. More than 60 percentage of the strains falls under *Salmonella enterica* subspecies, which includes two types of different group such as flagellated (H Antigens) and somatic (O Antigens) based on their serotyping results. Most of the infections (99%) caused by *salmonella* in humans and other mammals reported due to the enterica subspecies infections[2]. There are many epidemiological studies reported globally based on the phenotypical characteristics and biochemical reactions of *salmonella* spp. and PFGE is recognized to be one of the most precise and satisfactory method for studying the prevalence and genetic closeness of bacteria beyond their biochemical or serotyping results[6]. Many studies on PFGE profile of *salmonella* spp. have been documented around the globe [6, 7, 11, 17, 20]. A place like Maharashtra in India, there is very few studies carried out using PFGE methods[12], but more reports have been documented on the phage types and multidrug resistant studies in these area[14].In this study, isolation of *salmonella* bacteria is carried out using conventional methods. But for convenience, labour and reliability of confirmation of *salmonella* spp. RT PCR technique preferred over biochemical reactions. RT PCR methods are reported to reduce risk of cross contamination significantly and also produce a result which does not mislead while reading biochemical reactions using conventional microbiology steps[1, 3, 8, 14]. A possible false result in RT PCR method could be because of the presence of dead bacteria cells in samples and the DNA could be still detected. This issue is normally overcome by introducing an enrichment step of the test portion in Buffered Peptone Water or any other specific media for the pathogen under investigation and carrying out the DNA extraction next day ensures the results are only from viable cells [1, 12]. Contaminated water and food are the most common cause of diarrheal disease in India, and there are no systematic programs or strategies available for monitoring *salmonella* infections in humans and animals[9]. There are quite a number of epidemiological studies reported in India based on their antimicrobial susceptibility, biochemical reactions and serotype[10, 18, 13]. Further narrowing down to a state like Maharashtra, where there are not much paid attention in food quality control or agriculture and farming, such data are required in order to understand the prevalence and source of infections. The aim of this study will be to understand the genetic closeness and colonization of the *salmonella* spp in sewage water and soil in Nanded district, Maharashtra.

MATERIALS AND METHODS

1. ISOLATION

A total of 44 *salmonella* spp. isolates from environmental samples such as sewage and soil samples were included in the genetic diversity study. A standard protocol for *salmonella* isolation protocol (Based on ISO-6579:2002, Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. from World Health Organization (WHO) surveillance program was used in the study[7]. 25 grams of the sample was blended with Buffered Peptone Water (BPW, Oxoid) as primary enrichment at 35 ± 2^{0}C for 16-18 hours. A secondary enrichment was carried out by transferring 0.1 ml and 1 ml of the enriched BPW broth into 9.9 ml of Rappaport Vasilidias Broth (RVS, Oxoid), and 10 ml Muller-Kauffmann Tetrathionate Novobiocin (MKTTn, Oxoid) respectively. Broths were then incubated at 35 ± 2^{0}C for 16-18 hours and then streaked on to Xylose lysine deoxycholate agar (XLD, Oxoid) and incubated at 35 ± 2^{0}C for the same period as before. XLD plates were then examined for typical morphological characteristics of *salmonella* spp. and re-streaked on XLD plates again to purify the colonies if required. Single isolated typical *salmonella* colony was then streaked on to Nutrient Agar (NA, Oxoid) and incubated at 35± 2^{0}C overnight and identified using RT PCR.

2. DNA EXTRACTION AND IDENTIFICATION

For DNA extraction and identification, commercially available kits were used. For extraction, DNA Bacteria Plus (Qiagen, Germany) was used. 3 - 4 isolated colonies from NA plates from the previous step was aseptically transferred into the lysis tubes along with 400 µl of Cell Lysis Buffer (provided in the kit) and vortexed vigorously for 10 minutes. Tubes were then centrifuged at 14,000 rpm for 5 minutes and 100 µl of the supernatant was used as DNA starting material in RT PCR identification using Applied Biosystems MicroSEQ *Salmonella* spp. detection kit, and StepOnePlus instrument from Applied Biosystems. All positive isolates after RT PCR were stored for long term preservation in cryo-vials and used for PFGE analysis for studying the genetic diversity.

3. PREPARATION OF DNA PLUGS

All 44 isolates were subjected to PFGE analysis using a standardized one-day (24-26 hours) laboratory protocol from the Center Of Disease Control and Prevention (CDC), Atlanta, with slight modification. A standard *Salmonella enterica* serotype Braenderup H9812

(ATCC# BAA-644) was included as a reference standard in the study and processed as per the protocol along with the other isolated strains of *salmonella* spp. for PFGE analysis. Previously stored cultures in cryo-vials were revived by streaking them on to Trypticase Soy Agar (TSA, Oxoid) and incubating at 35 ± 2^{0}C. Cultures were then transferred in to a sterile Falcon tube (12 x 75 mm tube) contains 2 ml of the Cell Suspension Buffer (CSB, 100 mM Tris:100 mM EDTA, pH 8.0) using a sterile cotton applicator and adjusted the final turbidity to 1.0 – 1.5 range. 400 µl of the cell suspension transferred into a sterile 1.5 ml microcentrifuge tube and added 20 µl Proteinase K (20 mg/ml Stock). The tubes were left at room temperature for about 15 minutes. 1% Seakem Gold Agarose in Tris EDTA buffer (TE, 10mM Tris:1 mM EDTA, pH 8.0) was prepared and kept at 54^{0}C water bath. 400 µl of the melted agarose and 400 µl of the CSB in the microcentrifuge tubes were mixed gently using a micropipette and transferred immediately into the PFGE plug molds. Plugs were then allowed to cool down to room temperature for solidification.

4. CELL LYSIS AND PLUG WASHING

The prepared plugs were then transferred to 50 ml falcon tubes containing 5 ml of Cell Lysis Buffer (CLB, 50mM Tris:50mM EDTA, pH 8.0 + 1% Sarcosyl) with 25 µl of Proteinase K (Final concentration 0.1 mg/ml). Plug molds were incubated at 54 - 55^{0}C for 2 - 2.5 hours in shaking water bath (180 rpm). Later each plug was washed with 15 - 20 ml pre heated sterile water for 15 minutes with vigorous shaking in the water bath at 54 - 55^{0}C. Washing continued for 2 times with preheated water and 4 times with pre heated TE buffer.

5. DNA RESTRICTION DIGESTION

For each isolate only half portion of the prepared plug was used. Each of these portions were transferred to a sterile microcentrifuge tube after cutting, containing 200 ml of pre-restriction buffer (180 µl of Ultrapure Water (CLRW) and 20 µl 10X NE4 buffer, NEB). Tubes were left at room temperature for 10-15 minutes. Using a micro pipette the pre-restriction buffer was aspirated out carefully without damaging the plugs. Then 200 µl Enzyme master mix (176 µl CLRW, 20 µl 10X NE4 buffer, and 3 µl X*bal* (NEB, 20,000 U/ml)) was added into the tube and incubated at 37.5^{0}C in water bath for 2 - 2.5 hours.

6. GEL CASTING AND RUNNING

A total of 2.5 liters of 0.5X Tris Borate EDTA Buffer (TBE) (BIO-RAD) was used as running buffer. 100 ml 1% SeaKem Gold Agarose in 0.5X TBE buffer was prepared and left at 55- 60^{0}C water bath for casting. The restricted plugs were removed from water bath and aspirated out the restriction buffer and added 200 µl of 0.5X TBE and incubated at room temperature for 5 minutes. Plug slices were then loaded on the comb and poured the gel into the casting tray and left to set for 20 minutes. CHEF-DR III, BIO-RAD was used for running PFGE. An initial switch time of 2.2 second, Final switch time of 63.8 second, voltage of 6 V, included angle at 120^0 and run time set to 20 hours in 14^{0}C TBE buffer were used as running conditions.

7. GEL ANALYSIS

Once the run is finished, the gel was stained using 40 µl of ethidium bromide solution (BIO-RAD, 10 mg/ml stock solution) in 400 ml of CLRW for 30 minutes. Gel was de-stained for 45 minutes with repeatedly changing the distilled water. Gel image was taken using BIO-RAD GelDoc XR system under UV light. Band patterns on the gel was analyzed using Applied Maths, Bionumerics Software version 7.2, with the Dice coefficient (Tolerance of 1.5% and optimization of 1.5 %) and the dendrogram clustering was based on the default setting of UPGMA (Upweighted Pair Group Method Algorithm) settings. X*bal* digestion of *Salmonella ser. Braenderup,* H9812 was used as reference for analysis.

RESULTS AND DISCUSSION

Figure 1
Dendrogram showing the Xbal restriction pattern of 44 Salmonella spp. isolated form sewage and soil samples.

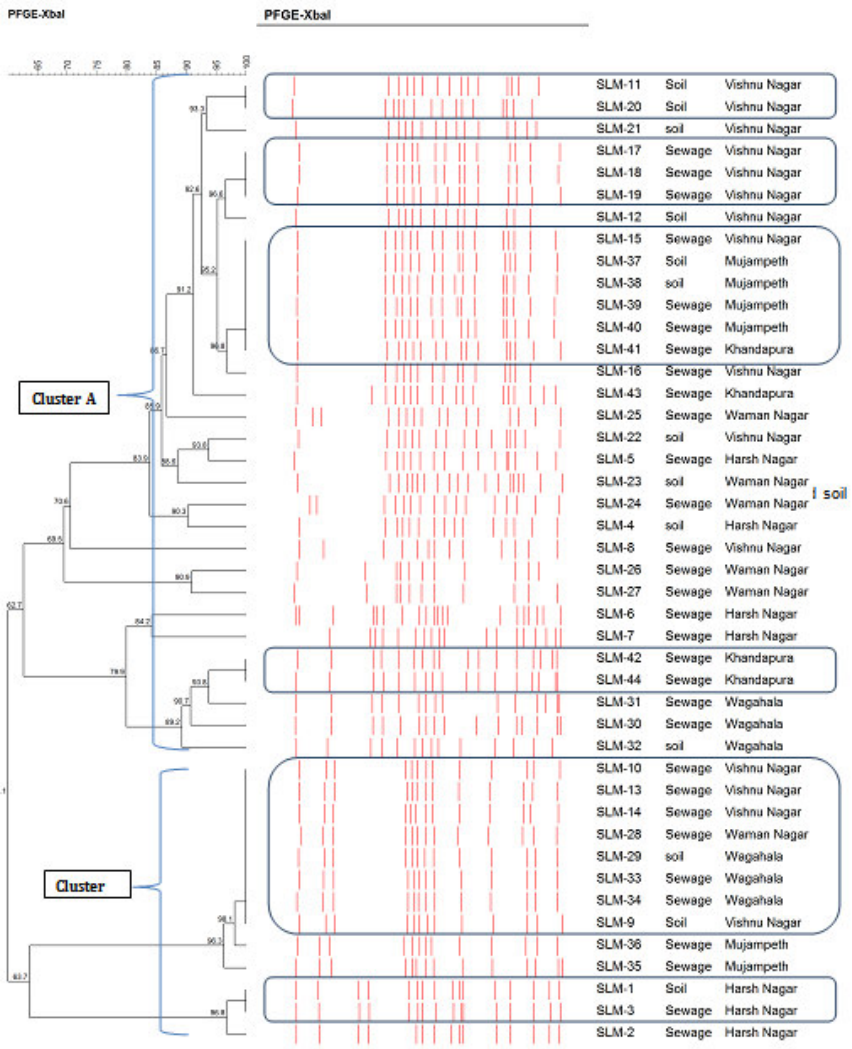

Table 1
Band pattern distribution of *Salmonella* isolates from soil and sewage sample

Place Name	Cluster A					Cluster B		
	G- 1	G- 2	G- 3	G- 4	Others	G- 1	G- 2	Others
Vishnu Nagar	1	3	2	0	5	4	0	0
Mujampeth	4	0	0	0	0	0	0	2
Khandapura	1	0	0	2	1	0	0	0
Wagahala	0	0	0	0	3	3	0	0
Waman Nagar	0	0	0	0	5	1	0	0
Harsh Nagar	0	0	0	0	4	0	2	1
Total	**6**	**3**	**2**	**2**	**18**	**8**	**2**	**3**
	31					**13**		

Table 2
Percentage distribution of salmonella isolates from soil and sewage sample

Place Name	Sample Type		Total Number	Percentage (%)	
	Soil	Sewage		Soil	Sewage
Vishnu Nagar	6	9	15	40.00	60.00
Mujampeth	2	4	6	33.33	66.67
Khandapura	0	4	4	0.00	100.00
Wagahala	2	4	6	33.33	66.67
Waman Nagar	1	5	6	16.67	83.33
Harsh Nagar	2	5	7	28.57	71.43
Total	**13**	**31**	**44**	**29.55**	**70.45**

There are two main clusters observed in the dendrogram (Figure 1), cluster A and cluster B. Most of the strains (31 out of 44 strains) isolated from the environmental sample are clustered together in the cluster A, whereas the cluster B consist of 13 environmental strains only.Cluster A has 4 groups of strains, which exhibits an indistinguishable band pattern. The major group is with 6 isolates including SLM 15, 37, 38, 39, 40, and SLM 41. The first and last strains of this group are from Vishnu Nagar and Khandapura area respectively and both are of sewage origin. All other 4 strains in the first group are from Mujampeth area and they are of both soil and sewage origin. SLM 37 and 38 are sewage isolates and SLM 40 and 41 are from soil samples. SLM 16 shares 96.8 percent similarity to the first group of isolates. SLM 16 is isolated from Vishnu Nagar area and from sewage source. The second group which exhibits 100% band pattern similarity are SLM 17, 18 and 19 which are all from Vishnu Nagar and all three are from the sewage samples. Another single strain (SLM 12) isolated from the same locality, Vishnu Nagar and from soil source shows 96.6 percent similarity to the second group in cluster A. The third group has two isolates, SLM 11 and SLM 20 with indistinguishable band pattern, and they both are from the same locality and same source. The third group shares a very close band pattern of 93.3 percent with SLM 21, which is also isolated from soil and same locality as Vishnu Nagar. The fourth group of strains also has two isolates, SLM 42 and SLM 44, both from same locality; Khandapura and they are from sewage origin. SLM 31 and SLM 30 strains isolated from Wagahala from sewage shares 93.8 and 90.7% similarity respectively to the group 4 in cluster A. Other strains are speeded over the cluster with a maximum similarity of 93.9% between SLM 26 and SLM 27, and a slightly less similarity of 93.3 % between SLM 22 and SLM 5. Another two isolates SLM 24 and SLM 4 shares 90.3% band pattern. SLM 43, 25, 23, 8, 6, 7, 30 and 32 are isolates speeded over the cluster A. with similarity to the major groups with less than 83%. The isolates SLM 43 share a 91.2% similarity to the other major first 3 groups in the cluster A, where SLM 25 shares only 86.7%. SLM 23 has a slightly lesser similarity of 85.9% to the main three groups of cluster A when compared to SLM 25. SLM 8 shares only 70.6% band pattern to any other groups of the cluster A. SLM 6 and 7 shares 84.2% similarity in their band pattern. There are two groups of environmental strains seen in cluster B which exhibits indistinguishable band pattern. The first group with 8 environmental strains which includes SLM 10, 13, 14, 28, 29, 33, 34 and SLM 9 and the second group with only two strains include SLM 1 and SLM 3. There are other two strains, SLM 36 and SLM 35 which exhibit 98.1 and 96.3% similarity respectively to the first group. Considering the large group in cluster B, majority of the similar strains are isolated from the sewage samples from three different localities, Vishnu Nagar, Waman Nagar, and Wagahala. The group includes only two strains isolated from soil sample, which are SLM 29 and SLM 9, which are from Wagahala and Vishnu Nagar respectively. SLM 35 and SLM 36 are of from Mujampeth locality and both were isolated from sewage samples. Also one strain, SLM 2 shows 96.8% band pattern similarity to the second group in cluster B. According to trenover criteria these three strains could be considered of the same origin, as they are only different in the one or two bands in the dendrogram analysis[19]. All three strains SLM 1, 2, and 3 are obtained from the same locality, Harsh Nagar. SLM 2 and 3 are from sewage samples and SLM 1 was isolated from soil sample.

CONCLUSION

The genetic diversity study using PFGE among *salmonella* isolates from various sewage and soil samples collected around Nanded district has shown the diversity and similarity of the genetic structure of the bacteria are spread over the place with similar and non similar genetic pattern. It is evident from the study that the numbers of the genetically identical isolates were more from the sewage than the soil samples and similar colonization of the *salmonella* spp. were from the sewage source. These data could be useful for identifying and studying the pathogen transmission pathways of future outbreaks and infections.

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