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Research Article

Genetic relatedness of Salmonella isolated from different poultry products in Nanded, (MS) India

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Abstract

This study was conducted in order to study the genetic relatedness and colonization of *salmonella* spp. isolated from various poultry products across Nanded district of Maharashtra. A total of 250 poultry products were included from six different localities of Nanded in the study. 19.2 % of samples were contaminated with *Salmonella* spp. The Pulsed-field Gel Electrophoresis (PFGE) subtyping technique was used to analyze the genetic diversity of all the *Salmonella* spp. isolated. PFGE study of the X*bal*-digested whole genome was analyzed using Bionumerics software using standard default settings. 3 major clusters of *Salmonella* spp. were observed in the final dendogram. 46 isolates produced a band pattern and out of which 15 *salmonella* spp. isolates showed indistinguishable band pattern in three clusters. In the first cluster 3 of them showed 100% similarity and 7 isolates in second cluster and 5 in the third cluster. The most number of *salmonella* spp. isolates with the same genetic origin was observed in cluster 3. The final conclusion indicates the spread of these isolates from all three different clusters to all 6 localities of Nanded district. To our best knowledge this is the first study conducted in comparing the genetic similarity and diversity of *salmonella* spp. isolates from poultry products in Nanded and these data could be useful in outbreak investigation and molecular surveillance program in the future.

INTRODUCTION

Salmonella spp. belongs to the family *Enterobacteriaceae* and all the members of this genus are Gram negative, non-spore forming, flagellated and facultative anaerobic bacteria. These mesophilic bacteria are with optimum growth temperature between 35 to 37^{0}C and a growth range of 5^{0}C to 46^{0}C. Pasteurization is an effective way of killing *salmonella* bacteria, which are highly sensitive to a pH below 4.5 (Amagliani *et al*., 2011; Dunkley *et al*., 2009). *Salmonella* are usually found in the intestinal tract of mammals and avian species, more predominantly in poultry (Ryan and Ray, 2004). Salmonellosis is food borne and it is considered as one of the major issues facing worldwide. About 95% of infections caused by salmonellosis are responsible for over 600 deaths and 1.4 million illnesses in the U.S. annually and the estimated cost of medical treatment and loss of productivity is reported to be around 500 million to 2.3 Billion USD (U.S FDA, 1996). Ingestion of contaminated water and food causes non-typhoidal salmonellosis and the major sources for this in the developed countries are poultry products (D'Aoust, 1994; Dunkley *et al*., 2009; Xia *et al*., 2009). The most important species of *salmonella* which cause human infection is *Salmonella enterica* and this was found from the recent studies on DNA

Hybridization of *salmonella* spp. Except S. typhi, and S. paratyphi all other genus are found in animals and humans. *S. enterica* causes salmonellosis and the most common source of this includes poultry and their products (Dunkley *et al*., 2009).In recent times, contaminated chicken and eggs are the most commonly identified vehicles which play important role to cause human Salmonellosis, as they have been responsible for pandemic associated with *S. enteritidis* between the mid 1980s and mid 1990s. Many outbreaks implicating poultry meat as the source of infection have been reported. In a study conducted at abattoirs in Canada, the prevalence rate of *Salmonella* in chicken was found to be 32.5% (D'Aoust, 1991). In many instances *salmonella* spp. are also isolated and reported from many dairy products and its derivatives (Poppe and John, 2011).

In India, *Salmonella* has been isolated from a variety of foods such as pork products, beef, fish and fish products, egg and as well as from water. The isolation rates from various foods varied from 0 to 100%. However exact magnitude of food borne Salmonellosis in India is not known yet known. Kumar, Y., 2009 and his colleagues carried out a study on *Salmonella* serovars and its distribution trend in India. A total of 1795 non-typhoidal isolates and 1284 typhoidal were identified in the year 2001-2005 in India. Out of 4672 isolates analyzed 14.4% were identified as *S. enteritidis* (Kumar *et al*., 2009). A study conducted in processed and unprocessed street foods for microbial profile of street food at Cochin, a heavily populated area in Kerala district in India, showed that the unprocessed street foods like Carrot juice and Watermelon were contaminated with *Salmonella* spp. and other pathogens. This is due to the poor handling and hygiene practices. Since these foods are consumed raw, in the forms of juices and fresh cuts, this could be serious issue in food related outbreaks and diseases (Poojara and Krishna, 2012). Narrowing it further to Maharashtra, a southern state of India where the food being imported from elsewhere and produced locally is not much paid attention for the quality and hygiene, resulting in multiple outbreaks throughout the year due to food poisoning (Sabiha and Niekar, 2012).

For research purposes and fast diagnostic study and analysis, conventional method of pathogen detection from various samples have been subjected must faster, automated and sensitive systems, like RT-PCR assays have been developed to reduce the laborious *Salmonella* isolation and serotyping steps (Handjinicolaou *et al*., 2008). Apart from saving time, RT- PCR is highly specific and sensitive and offers the potential for quantification. The risk of cross-contamination is significantly reduced, and high-throughput performance and automation are possible since no post-PCR manipulations are required. These novel real-time PCR assays were shown to be fast, sensitive, and reliable diagnostic tools that complement the existing toxin analysis methods. The developed assays could contribute substantially in determining the true incidences of the food poisoning caused by bacterial contamination especially by reducing the rate of misdiagnosis while reducing the time consumed for diagnostics by conventional microbiology (Amagliani *et al*., 2011; Danum *et al*., 2002; Jyoti *et al*., 2010; Riyas *et al*., 2004). The only limitation to these kinds of DNA based diagnostic methods is the possible detection or amplification of nucleic acid from non-living cells, leading to false positive results. Usually in food industries this problem is avoided by adding a culture-enrichment step to the food under investigation. This step will ensure the results are obtained from only viable cells (Amagliani *et al*., 2011).

Due to recent prominence of *Salmonella* species, a number of molecular typing methods have been used to improve the identification of this food-borne infection and also to differentiate strains above the level of serotyping (subtyping). Standard methods for identifying and typing of *Salmonella* spp. includes serotyping, biotyping, and phage typing, which may not be discriminative enough because more than 75% of the *Salmonella* isolated during an outbreak belonge to a single phage type (Xia *et al*., 2009).

In more recent years, the use of DNA-related techniques such as Pulsed-Field Gel Electrophoresis (PFGE), which is a DNA based whole genome fingerprinting method considered to be the "gold standard" for subtyping numerous bacterial pathogens because of its greatest discriminatory power and is considered a very strong genetic evidence based tracking platform. PFGE utilizes the whole genome DNA fingerprinting patterns to track the occurring food borne infections world-wide by utilizing standardized genotyping methods and sharing information in real-time through specific bioinformatics channels (Jyoti *et al*., 2010). There are many studies reported regarding the isolation and biochemical characteristics of *salmonella* spp.,

(Malorny *et al*., 2002; Martelli and Davies, 2012; Mercanoglu *et al*., 2009) however a thorough and detailed study in the genetic relatedness of these strains has to be evaluated in order to understand the source and the pathway of infections. There are very less studies carried or reported in India, especially in the state of Maharashtra (Sabiha and Niekar, 2012; Sangrulkar and Gandham, 2012).

MATERIALS AND METHODS

Salmonella isolation and identification

A total number of 250 poultry and poultry products, including raw eggs, fresh chickens, and slaughterhouse swabs were collected from 6 major places around in Nanded. A standard *salmonella* isolation method from World Health Organization surveillance program was used for isolation (Hendriksen, 2003). All samples were enriched using Buffered Peptone Water (BPW, Oxoid). 25 g of sample blended with 225 ml BBW and incubated overnight at 37^0 C. 0.1 ml of enriched broth then transferred to 10 ml of Rappaport Vasilidias Broth (RVS, Oxoid) and 1.0 ml into 10 ml Muller-Kauffmann Tetrathionate Novobiocin (MKTTn, Oxoid) broth and incubated 37^0 C overnight. The broth is then streaked on to Xylose lysine deoxycholate agar (XLD, Oxoid) and incubated overnight at 37^0 C. Typical *salmonella* strains from XLD are re-streaked to another XLD plate to purify the strains or any typical isolated colonies are streaked on to Nutrient Agar (Oxoid) and incubated overnight at 37^0 C. Strains from NA plates are stored into cryo-tubes for long-term preservation. All cultures from NA are identified using RT-PCR technique (Applied Bio system, RT PCR Step One Instrument, and MicroSEQ *Salmonella* spp. Detection kit) and positive isolates were used for studying the genetic relatedness using PFGE technique.

Preparation of DNA plugs

Standardized one-day (24-26 hours) laboratory protocol from Center Of Disease Control and Prevention (CDC), Atlanta with slight modification is used for *Salmonella* DNA fingerprinting. For making the plugs, *salmonella* strains from previously stored cryo vials are revived by streaking them to a Trypticase Soy Agar plate (TSA, Oxoid) and incubated at 35^0 C for 14-18 Hours. Cultures are then picked into 2.0 ml of Cell Suspension Buffer (CSB). The final turbidity is adjusted to 2.5 to 3.0 using bioMeriex Vitek colorimeter. 1% SeaKem Gold Agarose (SKG) in TE buffer is prepared and kept at 55-60^0C water bath until ready to use. 400 µl adjusted CSB is transferred to a micro centrifuge with 20 µl ProteinaseK. 400 µl of melted 1% SKG and 400 µl is gently mixed using a pipette and dispensed into plug molds. Standard *Salmonella enterica* serotype Braenderup H9812 (ATCC# BAA-644) was also included as the reference strain for the PFGE study.

Lysis of cells and washing of plugs

Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl) (CLB) was prepared. Each plug was then treated with 5 ml of CLB and 25 µl ProteinaseK in a 50 ml falcon tube (Final concentration of ProteinaseK in CLB calculated to be 0.1 mg/ml). Lysis process was carried out at 54-55^0C in a water bath for 3 hours with vigorous agitation. Plugs were then washed 2 times with 15 ml of preheated (at 54-55^0C) Ultrapure water and then 4 times with TE buffer.

Restriction digestion of dna in plugs

Half of the each plug was used for restriction digestion. Each plug was treated with 200 µl pre-restriction buffer (180 µl of Ultrapure Water (CLRW) and 20 µl NE4 buffer) for 15 minutes at room temperature. Then each plugs was incubated with restriction enzyme buffer of 200 µl (176 µl CLRW, 20 µl 10X NE4 Buffer, and 3 µl X*bal* (NEB, 20,000 U/ml)). Restriction digestion carried out at 37.5^0C in water bath.

Gel casting and running

2.5 liters of 0.5% Tris Borate EDTA (TBE) (BIO-RAD) running buffer was prepared and pre cooled to 14^0C. 1% SKG Agarose in 0.5 TBE buffer was prepared for casting the gel with 15 well combs. CHEF-DR III is used for running PFGE with initial switch time of 2.2 second, Final switch time of 63.8 second, Voltage 6 V, Included angle 120^0 and run time set to 20 hours in 14^0C TBE Buffer.

Gel analysis

After the run, gel was stained using 40 µl of ethidium bromide (BIO-RAD, 10 mg/ml) stock solution with 400 ml of CLRW in a covered gel-staining tray for 30 minutes with gentle shaking. Gel is then de-stained using CLRW for about 45 minutes, changing the water 3-4 times. A clear image of the gel was obtained using a BIO-RAD GelDoc XR system.

The gel was then analyzed used Applied Math, Bionumerics software (V.7.2). A dendogram was generated using Upweighted Pair Group Method Algorithm (UPGMA) default settings and Dice coefficient settings with a tolerance of 1.5% and optimization of 1.5%.

RESULT AND DISCUSSION

From 250 samples screened for *salmonella* species 48 of them were identified as positive for *salmonella* by RT PCR (Table 1). RT PCR identification is rapid, less laborious and sensitive method for the confirmation of bacterial strains (Eriksson and Aspan, 2007). Percentage of samples isolated from 6 different places varies in a range of 15 to 40% and with an average of 19.2% positive samples.

Table 1: RT PCR Analysis result for *Salmonella* spp identification.

Serial No	Place	Number of samples	Positive	Negative
1	Harsh Hagar	40	8	32
2	Mujamepth	40	3	37
3	Khadakpura	40	6	34
4	Vishnu Nagar	45	16	29
5	Waman Nagar	45	9	36
6	Waghala	40	6	34
Total		**250**	**48**	**202**

All *salmonella* positive isolates after RT PCR were characterized using PFGE with X*bal* enzyme digestion as per the standard PFGE protocol and from Except SLM 46 all other isolates produced a band pattern in the gel analysis after PFGE. This could be typical case of exonuclease activity where the DNA is completely degraded by exonuclease enzymes (Lewin, 2004). There are three clusters obtained in the dendogram. The most number of indistinguishable band pattern was observed with a total of 15 *salmonella* spp. isolates including, SLM 1, 2, 3, 4, 7, 10, 18, 34, 39, 40, 41, 42, 43, 44, 45. The first six strains were obtained from Harsh Nagar area. Strains SLM 43 to 45 were from Waghala. SLM strains 34, 39, 40, 41 and 42 from Waman Nagar, and SLM strain 18 from Vishnu Nagar. Isolates SLM 1, 2, 4, 7, and 10 were from Harsh Nagar. Although the strain SLM 18 and 34 produced 96.4% similarity and SLM 39, 3 and 40 showed 93.7% band pattern to other 10 strains in the group, which showed indistinguishable band pattern. According to Tenover criteria for chromosomal DNA restriction pattern the differences in two to three band position are considered to be included outbreak pattern if all epidemiological information are supportive (Barrett, 2006). Here in this scenario it is very evident that *salmonella* spp. of the same genetic origin are spreaded over the places. From the local survey it was found that all these chicken slaughterhouses were using the same chicken supplier who is available locally. The strains varies only in difference of one single band this could be due to a point mutation, which could create and lose a restriction site for the X*bal* enzyme, or a insertion or deletion of DNA from a fragment could cause the differences in the band number (Tenover and Arbeit, 1995). Similarly another major similarity band pattern between 12 *salmonella* isolates including, SLM 11, 12, 27, 6, 8, 9, 20, 21, 17, 19, 13 and 47 were observed in second cluster in the dendogram. The first seven isolates were with completely indistinguishable band pattern and the later five showed a 91.5% to the seven isolates. Also another small group of 3 isolates SLM 15, 28, and 5 shown indistinguishable band pattern in cluster one. In the same cluster another three isolates, SLM 22, 23, and 24 shared a 94% band pattern similarity. Except these three isolates in cluster one all other isolates do not share a considerable similarity in the genome structure. In table 2, the data indicates the diversity of the genetic structure of *salmonella* in different areas of Nanded. The most number of similar isolates were observed in cluster 3 with 19 strains spreaded in all 6 areas of study.

Foods borne infections are major concerns of the developing countries. This plays a major role in the economy of the country. It is very much clear from the fact that international trade and even local trade between different areas with in the same locality has increased tremendously over the years in order to meet the food requirement of the population (D'Aoust, 1994). The chances for the infection are increased same fold with these trade activities. So as the risk of infection increased with the absence of poor hygiene and quality standard systems practiced. Improper handlings of waste, and poultry feeds are also a major concern in remote areas. The pattern obtained from the gel analysis clearly shows that samples collected from different areas of Nanded have indistinguishable band pattern or of the same genetic origin and could pose danger of community outbreak of salmonellosis if not taken care properly.

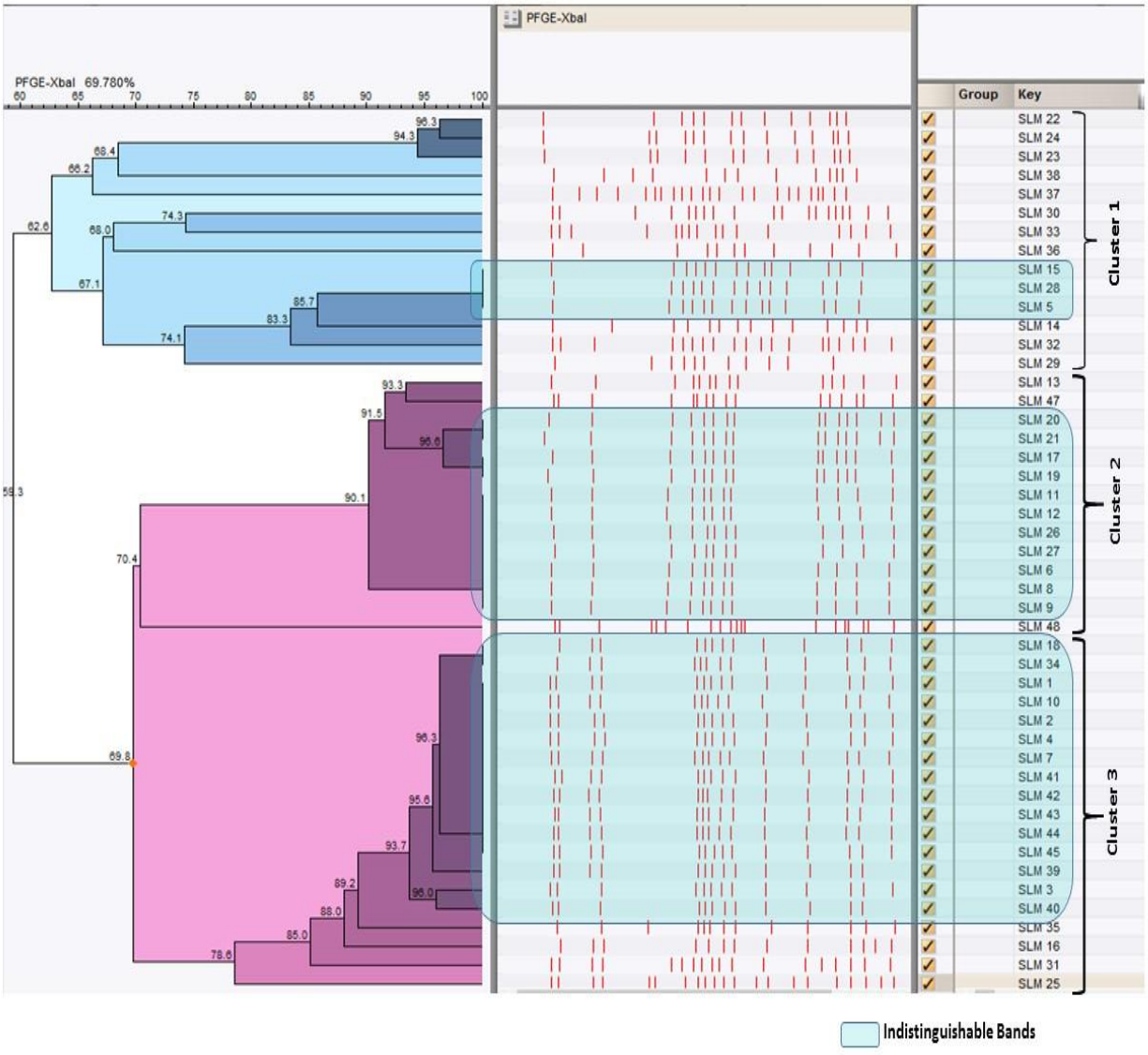

Figure 1: Dendogram- PFGE X*bal* pattern of 47 *Salmonella* spp. isolates from poultry products.

Table 2: Distribution of *Salmonella* spp. isolates in different clusters from 6 different areas of Nanded.

Serial Number	Places	Number of Isolates In		
		Cluster1	Cluster 2	Cluster 3
1	Harsh Nagar	1	2	5
2	Mujamepth	0	2	1
3	Khadakpura	2	3	1
4	Vishnu Nagar	8	5	3
5	Waman Nagar	3	0	6
6	Waghala	0	2	3

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