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Epidemiological Study on Shigella from Meat and its Public Health Significance

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A B S T R A C T

A study was designed to isolate and characterize the *Shigella* spp., from raw meat sold in Puducherry. A total of 150 raw meat samples (Chicken, chevon and beef each 50) were analyzed. Overall prevalence of 28(18.7%) isolates recovered and species were of *Sh.dysenteriae* and *Sh. flexneri*. Amongst 23 isolates maximum was recovered from chicken meat 14 (50%) followed by 7 (25%) chevon and 7 (25%) beef. *Sh. dysenteriae* was higher than *Sh.flexneri*. All the *Sh. dysenteriae* isolates were confirmed by PCR. Out of 14 PCR confirmed isolates of *Sh. dysenteriae*, 13 (92.8%) produced biofilm on modified congo red agar. In a total of 14 cultural and biochemical tests confirmed isolates of *Sh.flexneri*, 9 (64.2%) were of biofilm producers. Isolates showed maximum sensitivity against cefotaxime, azithromycin and cephalexin and complete resistance against amoxicillin and metronidazole. The high level of bacteria which could cause food borne illness in various foods presents public health risk to the consumer. This suggests the need to implement strict hygienic control measures along the food chain to improve the hygienic conditions during manufacturing, handling, storage and commercialization of foods.

Introduction

Nowadays, Shigellosis is one of the important foodborne illnesses that cause great menace to the public in developing countries like India. *Shigella* affects all age group people but very commonly in children and immune compromised individuals. *Shigella* consists of four species of Gram-negative, non-motile, non-spore forming, rod-shaped bacteria, namely *Sh. boydii, Sh. dysenteriae, Sh. flexneri* and *Sh. sonnei. Sh.dysenteriae* very commonly causes bacillary dysentery in human, characterized by mild to severe form of dysentery, fever, abdominal cramps and fluid loss. Virulence may vary with the strain (Ward and Hart, 1996). Infection mainly spread by eating contaminated food or by

drinking contaminated water (WHO, 2005). It has been reported that not less than 140 million cases of Shigellosis occur worldwide with 600,000 deaths annually and 60% of such deaths are seen in children below 5 years of age (HPA, 2004).

On the other side, an increased emergence of multi drug resistant *Shigella* also an equally challenging threat to the public. The persistence of organisms depends mainly on the production of tight covering of polysaccharides layers (Biofilm) related to the antibiotic resistance/disinfectant resistance (non-animate objects) (Dhanalakshmi *et al.,* 2015). Many studies have been documented on Shigella from meat nonetheless, public health significance was clearly meagre (Mukhopadhyay *et al.,* 2009; Iroha *et al.,* 2011; Ahmed *et al.,* 2013; Gayathri and Anu (2015); Ramya *et al.,* 2015; Rahimi *et al.,* 2016). Even though there are many studies on the microbial quality of fresh meat sold in Puducherry was under taken no systematic study has been conducted on the public health significance of *Shigella*. Therefore, present study mainly focuses on revealing the threat of *Shigella* and their characterization in most commonly consumed meats in and around Puducherry.

Materials and Methods

Chemicals and reagents

In this study all the chemicals, primers, reagents and culture media are procured from the Merck Limited, Hi media from Mumbai and Bangalore genei and PCR master mix was procured from Gene Technologies, Chennai.

Collection of meat Samples

A 100 gram of raw meat samples were collected from different retail meat shops in and around Puducherry (chicken, chevon and beef each 50) and subjected to this study. All the samples were collected in sterile polyethylene bags and immediately transported to laboratory in ice box for further analysis.

Methods of isolation and identifications

Twenty-five grams of each meat samples (chicken, chevon and beef) homogenized in 225 ml of Peptone Water (PW) and incubated at 37°C overnight. Pre-enriched culture inoculated into 9 ml of Selenite F Broth (SB) and Rappaport Vassiliadis Soya (RVS) Broth and incubated for 24 hours at 37^0C. Bacteria growing in SB and RVS broth were streaked onto Mac Conkey's agar for differentiation of lactose and non-lactose fermenters. Non lactose fermenter was streaked onto Xylose Lysine Deoxycholate (XLD) and Salmonella-*Shigella* agar (SS). The plates were examined for the presence convex colorless colonies on Mac Conkey's, colorless colonies on SS and XLD agar. The presumptive *Shigella* colonies were subjected to cultural, morphological and biochemical characteristics as per Cowan and Steel's, 1993.

Reference strain

The reference strain *Shigella dysenteriae* was obtained from Mahatma Gandhi Medical College and Research Institute, Puducherry. DNA was isolated by using boiling lysis method (Rahn *et al.,* 1994). Bacterial culture lysate prepared from the reference strain was used as template DNA for positive control in PCR.

Standardization of PCR for *Shigella dysenteriae* (*rfpB*)

The PCR was performed as per Ojha *et al.,* (2013) with slight modifications. Two sets of primer pair's species specific *rfpB* gene as shown in table no 1. A 25µl reaction mixture

was prepared in 0.2 ml thin-walled PCR tubes. The reaction mixture consists of the following: Template DNA - 5 µl, Primers (*rfpB*) - 20 pmol each primer, Master mix- 12.5 µl and Triple distilled water to 5.5 µl. Amplification was carried out in an automated thermal cycler (Eppendorf Mastercycler, Germany) according to the following programme. Initial Denaturation was at 94°C for 3 minutes followed by 30 cycles of Denaturation at 94°C for 30 seconds, Annealing at 60°C for 30 seconds, Extension at 72°C for 30 seconds and Final Extension at 72°C for 3 minutes.

Assessment of biofilm production of the isolates

Slime production assay was performed as per Vijayalakshmi and Lalitha, 2013. Briefly, Brain heart infusion agar supplemented with 5% sucrose and Congo red (0.08 g/l) was prepared and autoclaved. The isolates were inoculated and incubated aerobically for 24 to 48 hours. The ability of the isolates to produce bio-films was indicated by black colonies with a dry crystalline consistency and red colour colonies indicates negative.

Antimicrobial resistance profiling of the isolates

The drug susceptibility was performed by disc diffusion method in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2012). List of antibiotics given in table no. 2.

Results and Discussion

Slaughtering of livestock continues to increase, as a result of increase in demand for meat and its products (Warris, 2010) for human use. Its high consumption rate and popularity hence, makes contamination and its consequences as an issue of concern, since raw meat and meat products have been identified as potential vehicles of foodborne illnesses and implicated in food poisoning outbreaks (Bhandare *et al.,* 2007).

Food is an important vehicle for human infection of *Shigella* spp but the number of *Shigella* shedding from food is usually low. It is necessary to use pre-enrichment media such as peptone water or universal pre-enrichment broth to assist isolation. This may allow the small number of food or contaminated *Shigella*. Pre-enrichment normally helps to multiply or resuscitate *Shigella* that has been sub lethally damaged. Many studies have been documented the use of peptone water for pre-enrichment. In the present study peptone water used as a non-selectivepre-enrichment media. Peptone water was a preferred pre-enrichment media for Enterobacteriaceae family as it favoured the repair and growth of stressed or sub lethally injured isolates arising from exposure to heat, desiccation, high osmotic pressure or wide temperature fluctuations. (Ali *et al.,* 2010; Dabassa and Bacha, 2012; Zhao *et al.,* 2014; Makabanyane *et al.,* 2015)

Rappaport Vassiliadis Soy (RVS) broth and Selenite F broth (SB) were used as selective enrichment media. All the *Shigella* isolates only isolated from RVS. The other bacterial contamination also less compared to SB broth. And RVS broth also was found to be an effective enrichment media for preparation of template DNA for PCR examination compared to SB which did not produce good quality of DNA. Dabassa and Bacha (2012) used SB, RVS broth for secondary enrichment of *Shigella* and reported the less contamination with good number of isolates.

In this study, Mac Conkey's, XLD and Salmonella -Shigella agar were used. Mac Conkey's agar was used to differentiate non-lactose fermenting colonies and also purification of colonies from XLD and Salmonella - *Shigella* agar. Sangeetha *et al.,*

(2014); Mengistu *et al.,* (2014); Rezaee *et al.,* 2014 used MacConkey's, XLD and Salmonella-*Shigella* agar for the selective and differential isolation of *Shigella* isolates and suggested for the future studies. In present study MacConkey's agar, XLD and Salmonella-*Shigella* agar were helped to purify the *Shigella* colonies from contaminations. Results showed in table no. 3, 4 and 5.

Present study isolated *Sh. dysenteriae* and *Sh. flexneri* and isolated from all the three different raw meat available in Puducherry. No other species could able to recover. In chicken meat 14 (28%) isolates were recovered from 50 samples processed and screened for *Shigella.* Among the 14 isolates, 5 (37.7%) were *Sh.flexneri* and 9 (64.2 %) belonged to *Sh. dysenteriae*. Gayathri and Anu (2015) processed 50 chicken samples and the incidence of 2.5% *Sh. flexeneri* only was reported from Chennai, India. No other *Shigella* spp could be reported and also their incidence is comparatively less than present findings. Dhanalakshmi *et al.,* (2015) examined the chicken samples and 8% of *Shigella* was reported in Namakkal, Tamil Nadu. Their incidence is also less than our finding. In general incidence rates may differ according to place but the high bacterial contamination in the study may be attributed to the unhygienic practices during the slaughter of animals. This may be attributed to unclean wooden chopping blocks, improper sterilization of knife and other cutting equipment used and unhygienic person and personal habits, enhancing the chances of cross contamination of carcasses.

Out of the 50 chevon samples, 7 (14%) isolates were positive for *Shigella*. Among seven isolates four (57.1%) were of *Sh. dysenteriae* and three (42.8%) were of *Sh. flexneri*. Dhanalakshmi *et al.,* (2015) examined the chevon samples 5.8% (n=17) of *Shigella* was reported in Namakkal, Tamil Nadu. The increased incidence of *Shigella* indicates the suitable environment existing in the chevon shops. Increased contamination in meat shops arises maybe from the spilling of intestinal content over the carcass as well as meat cutting equipment and surfaces. This could also crossly contaminate the other carcasses which are slaughtered in the same place. Evisceration steps need to be focused for chevon to prevent the contamination in future.

A total of 50 beef samples screened for *Shigella*, seven (14%) isolates were recovered. Among the seven (14%) isolates *Sh. dysenteriae* (71.4%) was high than *Sh. flexneri* (28.5%). Rahimi *et al.,* (2016) carried out a study on *Shigella* species and detected *Sh. dysenteriae, Sh. flexneri* in meat, as well as the sources and contamination of isolates in Iran. In a total of 80 bovine meat samples analysed, frequency of *Shigella* species in beef was 8.7%. Among the *Shigella* isolates *Sh. flexneri* alone was reported around 6.8%. Were water considered main sources of contamination of *Shigella* in meat. Although overall prevalence of *Sh. flexneri* is higher compared to our study but the author has not reported any isolates of *Sh. dysenteriae*. But the present study isolated 10% of *Sh. dysenteriae* indicating the high level of this species contributed by contamination. Beef carcasses required more quantity of water for washing than other carcasses so, as the author reporter earlier that water may also be provided more chances of contaminations. Water samples can be selected for the isolation of *Shigella* so that the source of contamination may be traced or revealed.

Studies on *Sh. dysenteriae* were of less than other *Shigella* species. In Puducherry, majority of the dysentery mainly reported and diagnosed as due to *Sh. dysenteriae* (Mandal *et al.,* 2015). Our study main focus was given

to *Sh. dysenteriae* for the further confirmation. A total of 14 (18.7%) isolated *Sh. dysenterie* were subjected for PCR and all isolates were found to carry the *rfpB* gene. Ojha *et al.,* (2013) developed multiplex PCR for the detection of four *Shigella* spp in single reaction mixture. The incidence of *rfpB* gene was 100%. In this study application of *rfpB* gene detection was applied using conventional PCR and detected successfully. The details of results of PCR are depicted in diagram no. 1. Authors also illustrated that it is possible to detect *rfpB* gene from samples containing low bacterial concentration of *Sh. dysenteriae* by pre incubating the samples in growth medium. Because of rapidity, high specificity and sensitivity of PCR method, it is considered as better alternative to conventional culture method for confirmation of *Sh.dysenterie* especially in raw meat. Molecular detection of *rfpB* from *Sh. dysenteriae* indicates meat can be a source for the Shigellosis outbreaks in Puducherry.

A total of 28 *Shigella* isolates were recovered from the raw meat and 20 (71.4%) isolates were found to produce biofilm. In the study on biofilm for *Shigella*, 46.4% was contributed by *Sh. dysenteriae* and 25% from *Sh. flexneri*. Amongst both *Shigella* species *Sh. dysenteriae* was strong biofilm producers. This is in agreement with the findings of Quadri *et al.,* 1988 who studied the congo red uptake from solution by the different *Shigella* species and also reported the severity of the disease mainly mediated by virulence of *Shigella* species. In their results *Shigella* showed a distinct decline of binding in the order *Sh. dysenteriae*, *Sh. flexneri*, *Sh. boydii*, and *Sh. sonnei*. Dhanalakshmi *et al.,* (2015) detected the biofilm producing ability of meat isolates from Namakkal, Tamil Nadu. Out of 30 meat samples subjected for slime production assay on modified biofilm agar, *Shigella* spp produced biofilm of 33.3% from chicken meat and 100% of chevon samples, respectively. In the present study isolates were found to produce biofilm, it may be due to various environmental adaptation and mutational changes of the isolates. These isolates may be shows resistant to many antibiotics and disinfectant agents in future.

The emergence of multi drug resistant *Shigella* has remained a cause of concern in endemic regions (Thirunarayanan *et al.,* 1993). The details of the results and consolidated antimicrobial sensitivity pattern of 15 antibiotics depicted in table no. 6and7. Prevalence of antibiotic resistant nowadays an increased threat to the public. Out of the five isolates from chicken, 100% were sensitive to cefotaxime and chloramphenical, 83.4% were sensitive to cephalexin. Maximum isolates from chicken were showed resistant against co trimoxazole compound. This indicates being continuously used in chicken. Out of nine isolates of *Sh. flexneri* in chicken, 100% were sensitive to cephalexin, ciprofloxacin, chloramphenical, cefotaxime and tetracycline, 87.5% were sensitive to gentamicin, 100% Co-trimoxazole considered being the primary drug for the Shigellosis. Many authors have documented the resistant of co- trimoxazole and gentamicin isolates (Ashtiani *et al.,* 2009; Mengistu *et al.,* 2014 and Ocean *et al.,* 2015). In this study also 37.5% of the isolates were co-trimoxazole resistant. The emergence of multi drug resistance (MDR) is considered as a main factor for the treatment failure which is due to antibiotics used as under dosage or over dosage both in animals and human treatment which challenges further treatment by putting pressure on the selection of antibiotics for the treatment of *Shigella* infections.

Among the chevon isolates, 100% were sensitive to chloramphenical, cefotaxime and sulphamethizole, 75% were sensitive to tetracycline.

Table.1 Detail of the Oligonucleotide primer

Organisms	Target gene	Primer sequence (5'- 3')	Expected Product size (bp)	Reference
Shigella dysenteriae	*rfpB*	**F**:TCTCAATAATAGGGAACACAGC **R**:CATAAATCACCAGCAAGGTT	211	Ojha *et al.* (2013)

Table.2 List of antibiotics used in this study

Sr.No	List of antibiotics	Content/disk
01	Amoxycillin	10µg
02	Azithromycin	15µg
03	Co – trimoxazole	30µg
04	Cephalexin	30µg
05	Ciprofloxacin	30µg
06	Chloramphenical	30µg
07	Cefotaxime	10µg
08	Gentamicin	120µg
09	Metronidazole	50µg
10	Norfloxacin	15µg
11	Nalidixic acid	30µg
12	Perfloxacin	5µg
13	Sulphamethizole	30µg
14	Trimethoprim	25µg
15	Tetracycline	30µg

Table.3 Overall Prevalence of *Shigella*

Organisms	Chicken (n=50)	Negative Samples	Chevon (n=50)	Negative Samples	Beef (n=50)	Negative Samples
Sh. dysenteriae	5(10%)	45 (90%)	4 (8%)	46 (92%)	5(10%)	45 (90%)
Sh. flexneri	9 (18%)	41 (82%)	3 (6%)	47 (94%)	2 (8%)	48 (42%)

N : Total no. of samples

Table.4 Primary and secondary identification tests

Sr. No.	Parameters	Results
1	Shape	Rod
2	Motility	Negative
3	Catalase	Positive
4	Oxidase	Negative
5	Urease	Positive
6	Simmon's citrate	Negative
7	Indole	Negative
8	MR Test	Positive
9	VP Test	Negative
10	H_2S production in TSI slant	Negative
11	Lysine decarboxylase	Negative
12	Ornithine decarboxylase	Negative
13	ONPG	Positive

Table.5 Sugar fermentation results

Sr .no	Sugars	*Shigella*
1	Acid from	Positive
2	Dextrose	Neagtive
3	Dulcitol	Neagtive
4	Maltose	Neagtive
5	Xylose	Neagtive
6	Mannitol	Positive (only *Sh. flexneri*)

Table.6 Consolidated sensitivity pattern of *Sh. dysenteriae* (n=14)

List of antibiotics	Chicken (%)			Chevon (%)			Beef (%)		
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Amoxycillin	66.6	-	33.4	75	-	25	80	-	20
Azithromycin	-	33.4	66.6	-	25	75	-	-	100
Co – trimoxazole	100	-	-	75	25	-	80	20	-
Cephalexin	16.7	-	83.4	-	50	50	60	-	40
Ciprofloxacin	50	-	50	50	-	50	-	-	100
Chloramphenical	-	16.7	83.4	-	-	100	-		100
Cefotaxime	-	-	100	-	-	100	-	-	100
Gentamicin	66.7	-	33.4	100	-	-	100	-	-
Metronidazole	83.4	16.7	-	100	-	-	100	-	-
Norfloxacin	66.6	16.7	16.7	25	50	25	40	25	60
Nalidixic acid	50	33.4	16.7	50	25	25	-	-	100
Perfloxacin	50	40	50	50	25	25	40	40	20
Sulphamethizole	50	33.4	16.7	-	-	100	-	-	100
Trimethoprim	50	-	50	50	-	50	60	-	40
Tetracycline	50	-	50	25	-	75	60	-	40

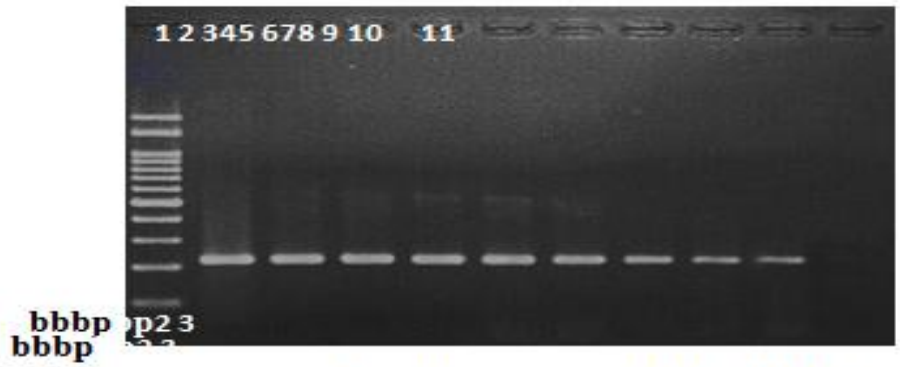
R - Resistant, I - Intermediate, S- Sensitive

Table.7 Consolidated sensitivity pattern of *Sh. flexneri* (n=14)

List of antibiotics	Chicken (%)			Chevon (%)			Beef (%)		
	R	I	S	R	I	S	R	I	S
Amoxycillin	100	-	-	100	-	-	100	-	
Azithromycin	-	-	100	33.4	66.6	-	-	-	100
Co – trimoxazole	37.5	12.5	37.5	-	-	100	-	-	100
Cephalexin	-	-	100	-	-	100	-	-	100
Ciprofloxacin	-	-	100	-	-	100	-	-	100
Chloramphenical	-	-	100	-	-	100	-	-	100
Cefotaxime	-	-	100	-	-	100	-	-	100
Gentamicin	-	12.5	87.5	-	-	100	-	-	100
Metronidazole	100	-	-	100	-	-	100	-	-
Norfloxacin	37.5	25	37.5	-	-	100	-	-	100
Nalidixic acid	25	-	75	-	-	100	-	-	100
Perfloxacin	62.5	-	37.5	33.4		66.6	-	-	100
Sulphamethizole	25	-	75	33.4	-	66.6	-	-	100
Trimethoprim	37.5	-	62.5	66.6	-	33.4	-	-	100
Tetracycline	-	-	100	-	-	100	-	-	100

R - Resistant, I - Intermediate, S- Sensitive

Picture.1 Standardization of PCR for detection of *Shigella dysenteriae*(*rfpB*)



Gentamicin resistant isolates were recovered and usage of gentamicin may be a misusage in goats. Out of three isolates of *Sh. flexneri* in chevon, 100% were sensitive to co-trimoxazole, cephalexin, ciprofloxacin, chloramphenical, cefotaxime, gentamicin, norfloxacin, nalidixic acid and tetracycline, out of five isolates of *Sh. dysenteriae* from beef, 100% were sensitive to azithromycin, ciprofloxacin, chloramphenical and cefotaxime, 100% were resistant to gentamicin and metronidazole. All the isolates were

equally shown resistance to amoxicillin and metronidazole compounds. In beef isolates all compounds were 100% sensitive.

This result is varying with Ahmed and Shimamoto (2015) who conducted a study on isolation and antibiogram of *Shigella* from raw meat in Egypt. Twenty four out of 27 (n=800) *Shigella* isolates (88.9%) were showing multidrug resistance to at least three classes of antimicrobials. The multidrug resistant *Shigella spp* were *Sh. flexneri* (66.7%), *Sh.sonnei* (18.5%), and *Sh. dysenteriae* (3.7%). The highest resistance was noticed to nalidixic acid (95.8%), tetracycline (95.8%), and sulfamethoxazole/ trimethoprim (87.5%). Mandal *et al.,* (2015) cultured the human diarrhoeal samples (n=210) in Puducherry for the presence of *Shigella* spp. Out of all the *Shigella* species isolated, *Sh. flexneri* comprised 90.5%, *Sh. sonnei* 5.4%, *Sh. dysenteriae* 2.7% and *S. boydii* 1.3%. The majority (79%) of the isolates of *Shigella* were resistant to co- trimoxazole, while 50% were resistant to ciprofloxacin. *Shigella* has become a public health concern because of the development of multiple antimicrobial resistant strains, emphasizing the importance of continuous monitoring of the pathogens. Resistance and reduced susceptibility to β lactam antibiotics is mainly caused by the production of CTX-M-type ESBLs (Zhang *et al.,* 2011). Vaccination seems to be the only sensible prophylactic approach for controlling shigellosis. Unfortunately, there is still no safe and efficacious vaccine available. Outer membrane vesicles are among the promising candidates to be used for vaccination against shigellosis (Camacho *et al.,* 2011). First generation virG based live attenuated *Shigella* strains have been successfully tested in phase I and II clinical trials and are a leading approach for *Shigella* vaccine development (Ranallo *et al.,* 2012). The presence of various pathogenic bacteria in different foods poses a health hazard and rise concerns about the safety of these food products. In addition, there is a need to implement strict hygienic measures in the manufacturing, handling, storage and selling of food in order to guarantee the quality of these foods so as to minimize or eliminate the risk of disease outbreaks. The presence of *Shigella* spp clearly shows the evidence of hazard in meat sold in Puduchery. And health officials and other public health sector need to focus on these aspects to avoid food borne outbreaks in future.

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