Identification of Biofilm Forming *Salmonella* Species of Layers from Tribal Area of Hoshangabad District of Madhya Pradesh

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ABSTRACT

The present experiment was designed to identify the biofilm forming *Salmonella* organism from cloacal swab, dry faeces, feed and water of layers in tribal area of Hoshangabad district, in rainy, winter and summer seasons. A total of 181 samples consisting of cloacal swab (109), dry faeces (18), feed (18), water (18) and egg shell (18) were collected in buffered peptone water in all the three seasons. For isolation, selective enrichment was performed in rappaport vassiliadis broth followed by streaking in macConkey, brilliant green agar, xylose lysine deoxycholate media. Phenotypically they were identified by motility followed by Grams staining and biochemical tests like oxidase, catalase, indole, methyl red, Voges Proskauer, triple sugar iron agar and urease. Further they were confirmed by latex agglutination test. Phenotypically identified 17 presumptive *Salmonella* isolates were subjected to genotypic characterization using 16S rRNA, *inv*A gene, *Salmonella enterica* serovar Typhimurium gene and *Salmonella enterica* serovar Gallinarum-Pullorum by PCR. All the isolates were positive (100%) for 16S rRNA and *inv*A gene, *Salmonella enterica* serovar Typhimurium gene were found to be 64.70%, while 35.29% isolates remain unidentified. Phenotypically and genotipically confirmed *Salmonella* isolates were subjected to biofilm formation. The percentagewise isolation of *Salmonella* organism from cloacal swabs were 14.63%, 5.26% and 10.00% in rainy, winter and summer season, respectively. While in rainy season from dry faeces, water samples and egg samples were 33.33%. The overall incidence of salmonellosis in layers from tribal area of Hoshangabad district was 9.39% (17/181) and all of them were biofilm producers.

Keywords: *Salmonella*, Hoshangabad, 16S rRNA and *inv*A gene.

Poultry are the important reservoir of many zoonotically important pathogens, of which *Salmonella* is of prime importance (European Food Safety Authority, 2010). Salmonellosis is one of the most important global poultry diseases which is caused by different *Salmonella* species (Kabir, 2010). *Salmonella* causes a large group of acute and chronic diseases in fowls and is the most important cause of mortality and morbidity in poultry. Occurrence of *Salmonella* in feed and feed ingredients is a well-recognized problem worldwide. This constitutes a considerable risk of *Salmonella* infection in poultry and subsequently in the consumers of infected products. Therefore, large resources are employed to control *Salmonella*. Still, some *Salmonella* isolates seem to be able to persist in the dry faeces, feed and environment for several years. The persistence of *Salmonella* is attributed to its capacity to form biofilm (Vestby *et al*., 2009). Conventional procedures offer a reliable method for differentiating *Salmonella* strains, but it is tedious and time-consuming. The development of PCR technology has allowed the specific amplification of particular target segment of DNA. Molecular methods are fast, as well as highly sensitive and very specific (Renu *et al.*, 2011).

Over five million people are directly or indirectly engaged in poultry sector, apart from numerous small poultry keepers in rural and tribal areas of the country. Hosangabad district of Madhya Pradesh occupies an important place

in tribal population. Here the tribes of 'gond' community keep poultry as supplementary income source through producer collectives. The resistant *Salmonella* strains can easily passed to human through food chains resulting in serious consequences in terms of treatment failure and rapid outbreaks of *Salmonella*. Hence, avian salmonellosis is of large economic concern in all phases of the poultry industry from production to marketing. So, the present experiment was designed to identify the invasive *Salmonella* organism from cloacal swab, dry faeces, feed and water of layers in tribal area of Hoshagabad district in all the three seasons.

MATERIALS AND METHODS

Collection of samples

A total of 181 samples consisting of cloacal swab (109), dry faeces (18), feed (18), water (18) and egg swab (18) were collected in rainy, winter and summer season from individual clinically ill birds.

Isolation of *Salmonella* organism

The *Salmonella* organism was isolated by conventional methods following methodology of Markey *et al.* (2013) with some modifications. Pre-enrichment was performed buffered peptone water incubating at 37°C for 18 h. Selective enrichment was performed in Rappaport Vassilidis media incubation at 42°C for 24 h and observed for turbidity for positive reaction. Subsequently the selective plating was done in MacConkey, brilliant green and XLD agar was inoculated and incubated at 37°C for 18 h. For the processing of feed samples the pre-enrichment was done in lactose broth at 37°C for 24 h.

Phenotypic identification

Phenotypically, for primary diagnosis Gram's staining and motility test was performed. Further identification was done by biochemical tests like oxidase, catalase, indole, methyl red, Voges Proskauer, triple sugar iron, urease test. Later they were confirmed by latex agglutination test.

Genotypic identification

All the phenotypically confirmed isolates were subjected to polymerase chain reaction (PCR) for 16S rRNA, *inv*A, *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Gallinarum-Pullorum gene using already published primers having annealing temperature 58°C, 60°C, 63°C and 65°C according to Stegniy *et al.* (2014) with some modifications. The initial denaturation was done at 94°C for 2 min, extension at 72°C for 40s and final extension at 72°C for 5min for 40 cycles. PCR reaction mix was 25 µL consisting of nuclease free water-6.5 µL, primermix 1, 2 and template- 2.0µL each and dream Taq™ Green PCR Master mix-12.5µL.

Biofilm formation assay

The persistence of *Salmonella* organism was confirmed by their biofilm forming capacity in 96 well micro titer plates. All the isolates of *Salmonella* species were sub cultured into Luria-Bertani (LB) broth individually and incubated aerobically at 37°C for 24 h and 48 h.

From each individual culture, 20 µl samples of in exponential phase and 180 µl of LB broth were dispensed in the wells of sterile 96 well flat-bottomed microtiter plates and kept for incubation at 30°C for 24 and 48 h. Each isolate was inoculated into at least 8 wells. The control well contained only LB broth without inoculation. After incubation, unbound cells were removed by inversion of micro titer plate, followed by vigorous tapping on absorbent paper. Subsequently, adhered cells were fixed with methanol. Adhered cells were stained by addition of 220 µl of crystal violet (0.5%) for 1 min. The stain was removed by exhaustive washing with distilled water. The plates were then allowed to dry. In order to quantify adhered bacteria, 220 µl of decolouring solution (ethanol / acetone, 80:20%) was added to each well for 15 min. The absorption of the eluted stain was measured at 590 nm. The strains were classified into the three categories: weak, moderate and strong biofilm producers as per Stepanovic *et al.* (2004).

RESULTS AND DISCUSSION

Isolation of *Salmonella* species by conventional method

For isolation of *Salmonella* species from layers, a total of 181 samples consisting of cloacal swab, dry faeces, feed, water and egg shell were collected in all the three

Table 1: Isolation of *Salmonella* species from layers by conventional method

Particulars	Cloacal swab			Dry faeces			Feed			Water			Egg shell			Total
Season	R	W	S	R	W	S	R	W	S	R	W	S	R	W	S	
Samples	41	38	30	06	06	06	06	06	06	06	06	06	06	06	06	181
Salmonella species isolated	06	02	03	02	0	0	0	0	0	02	0	0	02	0	0	17 (9.39%)
Percent positive	14.63	5.26	10	33.33	0	0	0	0	0	33.33	0	0	33.33	0	0	

R= Rainy, W = Winter, S = Summer

Table 2: Phenotypic characterization of *Salmonella* isolates

Sl. No.	Particulars	Motility	Oxidase	Catalase	Indole	Methyl Red	Citrate	Voges Proskauer	TSI	Urease	Latex Agglutination test
1	*Salmonella* species	+	_	_	_	+	+	–	+	–	+
2	Isolates positive	17	01	0	01	14	16	0	17	02	17
3	Isolates negative	0	16	17	16	03	01	17	0	15	0
4	Per cent positive	100	5.88	0	5.88	82.35	94.1	0	100	13.33	100

seasons viz. rainy, winter and summer. On the basis of conventional method 17 (9.39%) isolates were confirmed as *Salmonella* species.

Percentage of isolation of *Salmonella* species from cloacal swab were 14.63 % (06/41), 5.26% (02/38) and 10.00% (03/30) in rainy, winter and summer season, respectively. From dry faeces, egg shell and water sample percentage of isolation of *Salmonella* species were 33.33% in rainy season.

Phenotypic characterization of *Salmonella* species

All the samples were inoculated in buffered peptone water for pre-enrichment at 37°C for 18 h. after that inoculum was inoculated in to the rappaport vassiliadis media at 42°C for 24 h. The selective platting was done by inoculating in to the macConkey media and incubated at 37°C for 18 h. The transparent colonies were inoculated on brilliant green agar (BGA) at 37°C for 18 h. The pink colonies from BGA were again streaked on XLD media and incubated at 37°C for 18 h. All the black colonies (17 isolates) on XLD medium were suspected for *Salmonella*

organism and subjected to specific biochemical test for conformation. All the suspected isolates were stained with Gram's staining and pink coloured cocco bacilli were seen under 100X oil emersion in bright field microscope. The overall incidence of salmonellosis in layers from tribal area of Hoshangabad district was 9.39%.

A total of 17 isolates were phenotypically characterized by motility, oxidase, catalase, indole, methyl red, Voges Proskauer, triple sugar iron agar, urease and later confirmed by latex agglutination test. All the 17 isolates (100%) were positive for motility and TSI tests. The 1 isolates (5.88%) were positive for the oxidase test and indole test. The 10 isolates (82.35%) were positive for methyl red, 16 isolates (94.2%) positive for the citrate test and 02 isolates (13.33%) were positive for urease test. None of the isolates were positive for the catalase and Voges Proskauer test. The 17 isolates (100%) including catalase and Voges Proskauer negative isolates were positive for latex agglutination test by latex agglutination test kit.

In India, incidence and wide spread presence of *Salmonella* serovars of poultry origin is reported by many workers. Suresh *et al.* (2006) reported incidence of *Salmonella*

serovar on eggshell to be 5.9% and *S.* Enteritidis to be the major serotype. Savita *et al.* (2007) reported occurrence of *Salmonella* from diarrheic chickens and litter sample in Jabalpur, Madhya Pradesh with a prevalence of 8.69% and 3.22%, respectively. While in our studies the overall incidence of salmonellosis in layers from tribal area of Hoshangabad district was 9.39%. Another study revealed 4.82 % of chicken eggs were positive for *Salmonella* in north India (Singh *et al.,* 2010). *Salmonella* were isolated from 6.1% of environmental samples in West Bengal from backyard poultry (Samanta *et al.,* 2014). Poultry and eggs are considered as most important reservoirs from which *Salmonella* is passed through the food chain and ultimately transmitted to humans (Howard *et al.,* 2012). The feed which was supplied in this area to the layers was commercial feed having antibiotics as one of the ingredients, this may be the possible reason why we cannot isolate any *Salmonella* organism feed sample. Those poultry farmers who gave water untreated water to their flocks were at high risks of salmonellosis. Maximum *Salmonella* organisms were observed in rainy season. The higher prevalence observed dry faeces, water and egg shell in this study may perhaps be attributed to lack of awareness on the prevention and control of salmonellosis and poor management practices observed in most of the layer farmers. Conventional bacterial culture methods are still used most often to identify *Salmonella* and require at least 3-11 days. The standard culture methods for detecting *Salmonella* species in poultry include non-selective pre enrichment followed by selective enrichment and plating on selective and differential agars. These methods are time consuming and labor intensive.

Genotypic characterization of *Salmonella* species

A total of 17 phenotypically identified *Salmonella* isolates were further subjected for genotypic characterization using 16S rRNA and *inv*A gene by PCR. Isolates from cloacal swabs for all the 3 seasons for 16S rRNA and *inv*A gene were 100 % (17/17) positive. *Salmonella enterica* serovar Typhimurium gene were found 64.70% (11/17), while 35.29 (06/17) were remain unidentified. In present study the16S rRNA primers used for PCR amplification gave the band size of 550 bp in gel electrophoresis for all the 17 isolates. This band was detected in all the phenotypically characterized *Salmonella i*solates, indicating it as a genus specific primer. Our statement can be supported

by findings of Menghistu *et al.* (2011), they also got the PCR amplification of *Salmonella* isolates of approximate molecular size 550 bp.

In this study all the isolates were *inv*A gene positive showing band at 284bp in 1.2 % agrose gel electrophoresis. Previously, in a similar study Karmi (2013) detected *Salmonella* by conventional isolation methods and then PCR was carried out to detect *inv*A gene in isolated *Salmonella*. It was observed that 16% of samples were positive for *Salmonella,* 26% in meat samples and 6% in poultry samples. All the *Salmonella* isolates were positive for *invA* gene. AL-Iedani *et al.* (2014) isolated and identified *Salmonella* species from various sources of poultry farms by using four different techniques. In our studies also all the phenotypically characterized 17 isolates were later on confirmed by PCR. Contradictory to our findings, Sharma and Das (2016) conducted study to determine the *inv*A gene from chicken meat samples of North East area by bacterial culture method and PCR. Out of total 80 samples, 40 *Salmonella* isolates were found in chicken samples (50%) but all the *Salmonella* isolates were positive for *inv*A gene.

In PCR of the 17 isolates majority 11 (64.70%) of them were *S.* Typhimurium positive, showing band at 738 bp in 1.2 % agrose gel electrophoresis. Rychlik *et al.* (2014) and Ogunremi *et al.* (2017) also identified the two *Salmonella* serovar Enteritidis and Typhimurium, to be the most common serovars of causing food borne illness in Canada. In PCR of the 17 isolates, none of them were positive for *Salmonella enterica* serovar Gallinarum-Pullorum gene. The most probable reason could be that only single hatchery is located in the area of investigation. Once by conventional method they get the *Salmonella enterica* serovar Gallinarum-Pullorum positive sample reports they use to dispose of the bird. In tribal area of Hosangabad district chicks were supplied from the hatchery following biosecurity measures. So, no *Salmonella enterica* serovar Gallinarum-Pullorum could be found. According to Priyantha *et al.* (2008) developed countries have eradicated this serovar. Our data can also be supported by the fact that both fowl typhoid and pullorum diseases are caused by *Salmonella enterica* serovar Gallinarum-Pullorum occur frequently in Asian poultry. From 1996 to 2008 fowl typhoid was diagnosed in India, but the last outbreak of pullorum diease reportedly occurred in 2002 (OIE, 2010 a, b).

Table 3: Genotypic characterization of *Salmonella* species

Particulars	Cloacal swab			Dry faeces			Water			Egg shell			Total
	R	W	S	R	W	S	R	W	S	R	W	S	
16S rRNA	06	02	03	02	0	0	02	0	0	02	0	0	17
*inv*A gene	06	02	03	02	0	0	02	0	0	02	0	0	17
Salmonella enterica serovar Typhimurium	03	01	03	01	0	0	01	0	0	02	0	0	11 (64.70)
Salmonella enterica serovar Gallinarum-Pullorum	0	0	0	0	0	0	0	0	0	0	0	0	0
Unidentified *Salmonella* species	03	01	0	01	0	0	01	0	0	0	0	0	06
Total *Salmonella* species	06	02	03	02	0	0	02	0	0	02	0	0	17 (35.29)

R= Rainy, W = Winter, S = Summer

Table 4: Biofilm formation assay of *Salmonella* species

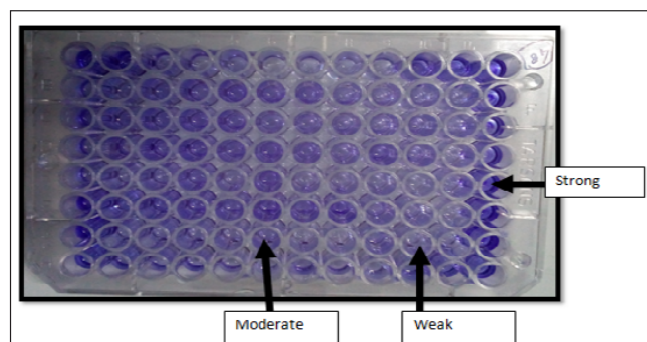| Total *Salmonella* isolates	Biofilm formation		
	Weak	Moderate	Strong
17	01 (5.88%)	02 (11.76%)	14 (82.35%)

Fig. 1: Biofilm forming assay of *Salmonella enterica* ser. Typhimurium

To check the persistence and invasiveness of *Salmonella* species all the 17 genotypically characterized isolates were subjected to biofilm forming assay. Biofilm forming assay was performed in 96 well microtitre plate at 24h and 48 h. There was no biofilm formation at 24 h. while, biofilm formation was recorded at 48 h. Out of 17, 16S rRNA positive *Salmonella* isolates were 5.88% (01/17), 11.76% (02/17) and 82.35%(14/17) strong, moderate and weak biofilm producers, respectively (Table 4).

In our study, at 24 hrs. of incubation none of the isolates were biofilm producers. After 48 hrs. of incubation, all the 17 *Salmonella* species isolates were biofilm producers and genotypically all of them were invasive. But different extent of biofilm formation was observed. Majority of our *Salmonella* species isolates were found to be strong biofilm producers followed by moderate and weak biofilm producers. In our study, LB broth (nutrient rich) was used for biofilm formation. Other investigators also reported that *Salmonella* species needs rich nutrients for biofilm formation (Stepanovic *et al*., 2004; Agarwal *et al*., 2011). The biofilm formation was recorded in all the isolates, samples irrespective of the source (cloacal swab, dry faeces, feed, and water and egg shell). Similarly the previous workers also reported that various factors (growth medium, incubation period, fixation of adhered cells and staining) affect development of *Salmonella* biofilm on microtiter plate (Stepanovic *et al*., 2004). According to those studies, the source of isolates (from humans, animals or food) did not affect the biofilm formation, which supports our study. Previously in a similar study, different extent of biofilm formation by *Salmonella* was observed among different serotypes. 151 strains of *Salmonella* consisting of reference and environmental isolates from diverse sources belonging to 69 serotypes were screened for biofilm production. Majority of strains (57.61%) were found to be moderate biofilm producers, while 34 (22.52%) and 29 (19.21%) strains were weak and strong biofilm producers, respectively (Agarwal *et al.*, 2011). Stepanovic *et al.* (2004) found 72.9% of the 121 *Salmonella* strains to be biofilm producers of which, 66.3% were strong biofilm producers. Different extent of biofilm formation by *Salmonella* was observed among different serotypes. In our study, all the isolates were positive for

*inv*A gene and 100% biofilm formation. This may be the reason for maximum being strong biofilm producers. Also, the source of *Salmonella* isolates does not seem to affect the ability to form biofilm on plastic surfaces (Stepanovic *et al.,* 2004). Different studies were conducted to compare the ability of different *Salmonella* serovar to produce biofilm on different surface (Agarwal *et al.*, 2011; Wang *et al.,* 2013). The optimized micro titer plate assay can be effectively used for the assessment of biofilm ability of *Salmonella* strains. Ability of majority of *Salmonella* strains to form biofilms on plastic surface as also shown in this study is of significance for food industry.

CONCLUSION

All the *Salmonella* isolates were showing positive for biofilm formation and are invasive in nature.

In rainy seasons incidence were high in cloacal swabs samples.

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