



Feed safety evaluation for prevalence of zoonotic *Salmonella* spp. in animal feed

V SHARMA¹, S SHARMA², A VERMA³, D K DAHIYA⁴ and M KARNANI⁵

Post Graduate Institute of Veterinary Education and Research, Jamdoli, Jaipur, Rajasthan 302 031 India

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ABSTRACT

Owing to the zoonotic nature of *Salmonella*, its transmission from feed-to-food is quite feasible and considered as one of the prime factors for the transmission and spread of virulent and drug-resistant strains in humans. Therefore, the present study was designed to investigate the prevalence of *Salmonella* in animal feed pellets from different feed mills in Jaipur and its nearby areas. For this, isolation of *Salmonella* was performed as per standard ISO methods and the presumable strains were further confirmed and characterized into different species by molecular methods. The confirmed strains were analyzed for virulence genes by PCR. Finally, the strains were analyzed for antimicrobial drug resistance by the standard disk diffusion method. The study revealed that the prevalence of *Salmonella* in feed pellets was moderate and *Salmonella enteritidis* and *Salmonella typhimurium* were the two dominated species. Strikingly, the majority of strains were found to possess the virulence genes and resistant to analyzed clinical antimicrobials. Results inferred that contaminated animal feeds may act as a potential source for the dissemination of virulent and drug-resistant *Salmonella* spp in animals as well as human beings. The present study implicated the need for more focused and extensive investigations from different parts of the country and the world for strict regulation of animal feed safety to assure one health concept.

Key words: Animal feed, Feed mills, PCR, *Salmonella enteritidis*, *Salmonella typhimurium*, Virulence genes

The role of animal feed in the production of safe food is recognized worldwide, and recent events have underlined its impacts on public health and food security. The presence of *Salmonella* in food or feed is of great public health concern and each year this pathogen causes a significant economic loss to the food industry (Magossi *et al.* 2018). Consumption of contaminated foods is attributed as the major source of infections not restricted to *Salmonella* (Sharma *et al.* 2017a; Sharma *et al.* 2017b) and its accidental ingestion leads to severe complications in the host. Animal feed is an insight as one of the important vehicles of *Salmonella* transmission not only in poultry and piggy industry but to cattle also and is a growing public health issue (Sharma *et al.* 2017c). Contamination of compound animal feed meant for livestock use by *Salmonella* has been available in the literature (Sharma *et al.* 2015), but the data is mainly restricted to western countries. In a feed mill, different factors impart contamination of *Salmonella* to feed (Burns *et al.* 2015). Shifting the material from one place to another, placing the material into intake pit and human interference creates a lot of dust that might carry *Salmonella* into the premises. Contamination can also occur by biological vectors (Pellegrini *et al.* 2015). Moisture and

temperature can also have a significant influence on the bacterial load of finished feed. Converting the ingredients in form of pellets might reduce the bacterial load of finished pellet but not completely eliminate their contamination. Furthermore, the recent rise in pesticide residue in food products and antimicrobial resistance among the foodborne pathogens has become a serious public health issue (Sharma *et al.* 2015, Sharma *et al.* 2016). Studies are available that have correlated the link between antibiotic use and the emergence of antibiotic-resistant microorganisms in animals and within the farm environment (Magouras *et al.* 2017). Therefore, the presence of *Salmonella* in animal feed should not be disregarded as food-producing animals are identified as the major source of *Salmonella* infections in humans. Keeping the above rationale in mind, the present study was undertaken to assess the prevalence of *Salmonella*; its virulence gene profile and antibiotic susceptibility in the animal (livestock) feed mills situated in Jaipur, India.

MATERIALS AND METHODS

Study design and sampling: From 35 feed mills of Jaipur city, a cross-sectional study was carried out in 2016. In all, 200 samples including 100 finished pellets and 100 mash samples were collected at different time points. Approximately 100 g sample was obtained in a sterile bag and further grounded to powder in a sanitized grinder. Samples were stored in a cool and dry place until bacteriological and biochemical analyzed for *Salmonella*.

Present address: ^{1,2}Professor (drvishnus@yahoo.com, drsanjitas@gmail.com), ³Teaching Associate (aagosh.minkss@gmail.com), ⁴Research Associate (dineshlucky31@gmail.com), ⁵Assistant Professor (dr.monikakarnanai@gmail.com).

Isolation and identification: Identification of *Salmonella* in feed samples was carried out according to the standard culture method; ISO – 6579: 2002 (Koyuncu and Haggblom 2009). Further colony morphology was determined by Gram staining procedure. The presumptive *Salmonella* colonies were confirmed and characterized in different species by *Salmonella* API 20E test kit.

Extraction of genomic DNA: The genomic DNA was extracted in accordance with a previous method (Dahiya and Puniya 2017), with fewer modifications. The cultures that were found positive during API analysis were processed for DNA extraction. The extracted DNA was quantified and analyzed for purity using a nanodrop reader. Afterwards, the eluted DNA was stored at –20°C in TE buffer.

Genus and species level characterization: The primers and programme mentioned in Table 1 were used for the genus and species characterization as elaborated previously (Oliveira *et al.* 2002). PCR reaction (25 µl) composed of 12.5 µl Green Master Mix, 0.5 µl of each primer (10 pmol), 1 µl of genomic DNA (500 ng/µl) as a template and nuclease-free water was used for amplification of specific primers in a thermal cycler. The amplified bands were visualized in a gel documentation system after performing gel electrophoresis.

Virulence gene characterization: The virulence genes identified in *Salmonella* isolates are listed in Table 1, and the primers and PCR conditions were in accordance with the previous findings (Smith *et al.* 2015). A 25 µl reaction composed of 12.5 µl Green Master Mix, 0.5 µl of each primer (20 pmol), 1 µl of genomic DNA (500 ng/µl) and

Table 1. List of primers

Primer	Primer sequence	Amplification product (bp)
Salm – 3	GCT GCG CGC GAA CGG CGA AG	389
Salm – 4	TCC CGG CAG AGT TC CAT T	
InvF/	ACA GTG CTC GTT	284
invAR	FTAC GAC CTG AAT	
InvR/	AGA CGA CG GTA CTG	
invar R	ATC GAT AAT	
139 (1)	GTG AAA TTA TCG CCA	284
	CGT TCG GGC AA	
141 (1)	TCA TCG CAC CGT	
	CAA AGG AAC C	
Fli 15	CGG TGT TGC CCA GGT	620
	TGG TAA T	
Typ 04	ACT GGT AAA GAT GGC T	
Sit C F	CAG TAT ATG CTC AAC	750
	GCG ATG TGG GTC TCC	
Sit C R	CGG GGC GAA AAT AAA	
	GGC TGT GAT GAA C	
spvA F	GTC AGA CCC GTA AAC AGT	604
spvA R	GCA CGC AGA GTA CCC GCA	
spvB F	ACG CCT CAG CGA TCC GCA	1063
spvB R	GTA CAA CAT CTC CGA GTA	
A058	GAT ACT GCT GAA CGT AGA AGG	488
A01	GCG TAA ATC AGC ATC	
	TGC AGT AGC	

Table 2. Results of API kit

	OX	ARA	AMY	MEL	SAC	RHA	SOR	INO	MAN	GLU	GEL	VP	IND	TDA	URE	H2S	CIT	ODC	LDC	ADH	ONPG
P1	SE			+	+			+		+	+						+				
P2	SE			+	+			+		+	+						+				
P3	SE			+	+			+		+	+						+				
M1	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
M2	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
P4	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
P5	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
P6	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
P7	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
M3	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
M4	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
M5	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
M6	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
M7	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
M8	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
M9	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
M10	ST	+		+	+		+	+	+	+	+						+	+	+	+	+
P8	ST	+		+	+		+	+	+	+	+						+	+	+	+	+

SE, *Salmonella enteritidis* and ST, *Salmonella typhimurium*.

10.5 µl nuclease free water was used for PCR amplification in a thermal cycler.

Antibiotic sensitivity pattern: All confirmed isolates were subjected to antibiotic susceptibility testing for 11 different antibiotics as ampicillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), imipenem (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), gentamycin (10 µg), streptomycin (10 µg), co-trimoxazole (25 µg), chloramphenicol (30 µg) and tetracycline (30 µg). The susceptibility of *Salmonella* isolates was determined by the agar disc diffusion method (Dahiya and Puniya 2015). After 16–18 h of incubation at 37°C the zones were interpreted as resistance, intermediate and sensitive in accordance with Clinical and Laboratory Standards Institute (CLSI 2015).

RESULTS AND DISCUSSION

Salmonella contamination of animal feed insight as one of the most probable causes of foodborne outbreaks (Magossi *et al.* 2018) and identified as the biggest threat to food safety. There is an enormous possibility that contaminated feed becomes a way for the entrance of this microorganism in the food chain (EFSA 2018). Exhaustive information on the prevalence of *Salmonella* in animal feeds is not available throughout the world provided the fact that animals are an integral part of our food chain system. Very scanty information regarding this issue is available from Indian sub-parts. Therefore, this study was designed to assess the prevalence, virulence potential, and antibiotic susceptibility of *Salmonella* isolates recovered from different cattle animal feed mills situated in Jaipur. Out of 200 samples (100 each from pellets and mash) examined, *Salmonella* was isolated from 12 pellet samples (12%) and 22 mash samples (22%) on the basis of specific media plating and microscopic analysis.

All 34 presumptively identified *Salmonella* cultures were further characterized as *Salmonella* by API 20E biochemical kit and species-specific PCR and delineated that they have composed of two different species; *Salmonella enteritidis* (04) and *Salmonella typhimurium* (30) (Table 2).

In the present study, moderate prevalence of *Salmonella* was observed. Contamination of animal feed is also documented from other parts of the world. Studies accomplished from Brazil, United States, and China indicates the incidence of *Salmonella* in animal feedstuffs varies and ranged from 0.66% to 13.1% (Burns *et al.* 2015, Pellegrini *et al.* 2015, Hsieh *et al.* 2016, Yang *et al.* 2017, Magossi *et al.* 2018). Various factors such as dust, storage conditions, labor interference, transport facilities, handling or processing into mixed feeds are insights as critical points for the contamination of animal feed by *Salmonella*.

All 34 *Salmonella* isolates (*Enteritidis* and *Typhimurium*) were found to possess the *invA* virulence gene that encodes for a protein which helps the bacterium to invade the host epithelial cell. The results in this concern coincide with earlier studies (Amini *et al.* 2010, Chaudhary *et al.* 2015), where similar findings were obtained because of the conserved nature of this gene. *Sit C* that encodes for iron

acquisition protein is found in 91.17% of isolates and is in contrast to the previous finding (Smith *et al.* 2015), that reported it in only 50% isolates. Similarly, gene *spv B* was present in 94.11% of samples and in agreement with Amini 2010 findings that had observed high prevalence (88.6%) for this gene. In the present study, *spv A* was only present in 2.94% of the isolates and the results are in contradiction with the findings of Amini 2010 that had noticed the higher prevalence, i.e. in 90% of the isolates (Table 3).

Antibiotic resistance in the food chain is considered as a serious health issue worldwide, and microbes present in food plays a vital role in spreading this resistance (Tang *et al.* 2017). Therefore, the susceptibility of *Salmonella* isolates recovered from cattle feed was analyzed and the results demonstrate that they are multidrug resistant. The most resistance was noticed for cepheems followed by penicillin and sulfonamide (Table 4). The results in this concern are in concordance with an earlier finding (Lopes *et al.* 2015), which observed that *Salmonella* isolates

Table 3. Positive samples for *Salmonella*, *S. enteritidis*, *S. typhimurium*, and virulence genes

	Inv 139 141	Fli C, typ	A058, A01 (Sef A)	Virulence gene specific primers		
				Sit C	Spv A	Spv B
P1	+	—	+	+	—	+
P2	+	—	+	+	—	+
P3	+	—	+	+	—	+
M1	+	+	—	+	—	+
M2	+	+	—	+	—	+
P4	+	+	—	+	—	+
P5	+	+	—	+	—	—
P6	+	+	—	+	—	+
P7	+	+	—	+	—	+
M3	+	+	—	+	—	+
M4	+	+	—	+	—	+
M5	+	+	—	+	—	+
M6	+	+	—	+	—	+
M7	+	+	—	+	—	+
M8	+	+	—	+	—	+
M9	+	+	—	+	—	+
M10	+	+	—	+	—	+
P8	+	+	—	+	—	+
P9	+	+	—	+	—	+
M11	+	+	—	+	—	+
M12	+	+	—	+	—	+
M13	+	+	—	+	—	+
M14	+	—	+	—	—	+
M15	+	+	—	+	—	—
P10	+	+	—	+	—	+
P11	+	+	—	—	—	+
M16	+	+	—	+	—	+
M17	+	+	—	+	—	+
M18	+	+	—	+	—	+
M19	+	+	—	—	+	+
M 20	+	+	—	+	—	+
M 21	+	+	—	+	—	+
P 12	+	+	—	+	—	+
M 22	+	+	—	+	—	+

Table 4. Overall antibiotic susceptibility patterns of *Salmonella* isolated from various processed cattle feed samples

Antimicrobial	Total number of isolates tested	Resistant N (%)	Intermediate N (%)	Sensitive N (%)
Tetracycline (T)	34	2 (6%)	0 (0%)	32 (94%)
Chloramphenicol (C)	34	1 (3%)	2 (6%)	31 (91%)
Co-Trimoxazole (COT)	34	4 (12%)	1 (3%)	29 (85%)
Streptomycin (S)	34	1 (3%)	4 (12%)	30 (88%)
Gentamicin (GEN)	34	2 (6%)	2 (6%)	30 (88%)
Ciprofloxacin (CIP)	34	3 (9%)	7 (21%)	24 (70%)
Nalidixic acid (NA)	34	5 (15%)	5 (15%)	24 (70%)
Imipenem (IPM)	34	2 (6%)	1 (3%)	31 (91%)
Ceftazidime (CAZ)	34	7 (21%)	7 (21%)	20 (59%)
Cefotaxime (CTX)	34	18 (53%)	10 (29%)	6 (18%)
Ampicillin (AMP)	34	5 (15%)	0 (0%)	29 (85%)

recovered from feed mills were mostly multidrug resistant and also depicts frequent resistance to sulphonamide. Nalidixic acid is a quinolone drug and resistance associated with it has been due to various point mutations in DNA gyrase enzyme where the drug acts. High sensitivity to chloramphenicol observed in the present study was corroborated to an earlier study, which signifies that the majority of *Salmonella* isolates analyzed by them were sensitive to chloramphenicol (Elmadiena *et al.* 2013). Furthermore, high susceptibility of isolates to carbapenem drug was in agreement with a previous investigation, where meropenem was found as an excellent therapeutic option for multidrug-resistant *Salmonella* isolates (Tang *et al.* 2017).

In conclusion, feed pellet and mash collected from feed mills were found contaminated with the *Salmonella* and may act as a source of transmission to humans via the food supply chain. Higher prevalence was noticed in mesh feed in comparison to pellet and *S. typhimurium* was identified as the predominant species. Molecular analysis revealed that virulence gene *spvB* was present in most of the isolates. Also, most of the strains were found as multidrug resistant. From here it is inferred that the cross-contamination of food by contaminated feed may transfer the infections in the society. However, this is only a preliminary study that emphasizes on the need for further investigations in this direction from other geographical regions of the state to get a clear cut picture about the prevalence of *Salmonella* and to assess the food safety risks.

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