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Isolation and prevalence of *Salmonella* serovars from poultry in different parts of Haryana, India*

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ABSTRACT

The present study was undertaken to elucidate the epidemiological status of various *Salmonella* spp. in poultry in Haryana, India. *Salmonella* isolates (88) were recovered from disease outbreaks in broilers from different regions of Haryana. Typical morphology and colony characters on MLA and BGA, biochemical reactions, serotyping, scanning electron microscopy, along with antibiogram profiles were able to group the isolates into 4 groups namely *Salmonella* Gallinarum (53), *Salmonella* Pullorum (16), *Salmonella* Enteritidis (13) and *Salmonella* Typhimurium (06). Report of isolation of *Salmonella* Pullorum is the first report from this region. Report of isolation of zoonotically important isolates is the cause of concern. Multiple drug resistant patterns were observed in most of the isolates which can hamper control of mortality in young chicks in future.

Key words: Poultry, Prevalence, Salmonella

Salmonellosis is an endemic disease in India affecting both man and animals alike. Although more than 2,300 serotypes of *Salmonella* have been identified, only about 10% of these were isolated from poultry (Kabir 2010). In India, more than 235 serovars have been recorded and this number is increasing constantly (Reeves *et al*. 1989). Salmonellosis in poultry caused by *Salmonella* Gallinarum, *Salmonella* Pullorum, and *Salmonella* Enteritidis are the leading causes of morbidity and mortality in poultry and responsible for significant economic losses.

Earlier, 95 isolates of *Salmonella* Enterica belonging to *S.* Typhimurium, *S.* Enteritidis, *S.* Gallinarum, *S.* Paratyphi B and *S.* Bareilly were obtained with an overall prevalence rate of 14.40% from North eastern region of India (Murugkar *et al.* 2005). Prakash *et al.* (2005) studied the prevalence of salmonellosis in Karnataka, Maharashtra and Tamil Nadu. The most predominant serotype was *Salmonella* Gallinarum accounting for 69.6% followed by *Salmonella* Enteritidis for 21.7%. Aim of present study was to know the prevalence of *Salmonella* serovars in poultry in different geographical regions of Haryana (India) and characterize these isolates based on culture characteristics, biochemical features, serotyping, scanning electron microscopy and drug resistance pattern to find out its epidemiological status.

MATERIALS AND METHODS

Study sites

The study was conducted mainly in Hisar and adjoining districts, viz. Jind, Bhiwani, Sirsa, Fatehabad and Rohtak regions of Haryana (India) in the year 2007–08.

Isolation of Salmonella *strains from different outbreaks*

Salmonella strains were isolated from dead birds presented for postmortem examination from different poultry farms of the region with the history of heavy mortality and characteristic postmortem lesions.

Identification of bacterial isolates, serotyping and biochemical characterization

Culture characteristics on MLA and BGA were used for initial identification of *Salmonella* spp. Bacterial colony from each culture was further stained by Gram's staining. Impression smear of liver from affected birds was also made on a clean glass slide and gram's staining as well as methylene blue staining was done for presumptive diagnosis**.** Agglutination was carried out with Salmonella positive serum to confirm the Salmonella isolates. Biochemical tests were performed as per O.I.E manual of standards for diagnostic tests and vaccines as well as with *Salmonella* identification kit. For motility test semisolid nutrient agar with 0.2–0.3% agar was used with cregy's tube kept inside. Culture was

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inoculated in the middle of cregy's tube and kept overnight at 37°C. If the growth appeared on the upper part of the nutrient agar then bacteria were characterized as motile, if the growth appeared on the site of inoculation only, then non-motile. Serotyping was performed as per the methodology adopted by National *Escherichia* and *Salmonella* Centre, Kasauli (Himachal Pradesh). Briefly 18 to 24 h broth culture of the test strain was taken and centrifuged at 2000 rpm for 15 min. Drop of centrifuged deposit was applied to a drop of O polyvalent group A-E antisera. Culture suspension was further tested with various group specific sera through groups A-E. H-antigens, phase 1 and 2 were determined using polyvalent diagnostic sera. Kauffman- white scheme was consulted and organisms were assigned to the particular *Salmonella* serotype.

Scanning electron microscopy

Single colony from fresh culture on BGA was inoculated in 5ml BHI broth in different centrifuge tubes and incubated for 24 h at 37°C. Centrifuged the broth at 1000 rpm for 10 min to get a pellet. Decanted the medium and washed the pellet with PBS, pH 7.2. The pellet was fixed with 10% glutaraldehyde (EM grade).Supernatant was removed and the pellet was washed with ethanol in increasing order (30, 40, 50, 60, 70, 80, 90%) for 5–7 min each and finally with absolute alcohol for 20 min. For complete drying, the samples were dipped in hexamethyl disilazane for 15 min. After complete drying, the samples were loaded onto grids and kept in dessicator for further drying overnight at room temperature. Next day, coating of the colloidal gold particles on the grid was done with the help of ion sputter at high voltage (12000 V). The grids were then examined in scanning electron microscope and photographed.

Antibiotic sensitivity test was performed as per the disc diffusion method (Bauer *et al.* 1996). Five different antibiotic discs namely chloremphenicol C (30 mcg), amikacin Ak (30 mcg), gentamicin G (10 mcg), cephotaxime Ce (10 mcg) and cephadroxil Cq (30 mcg) were used. Results were recorded using antibiotic zone scale and interpreted as resistant, intermediate and sensitive based on values given in zone size interpretative chart.

RESULTS AND DISCUSSION

Isolation

Impression smears of affected liver when stained with methylene blue revealed small bacterial rods under oil emersion suggesting the bacterial infection, which was confirmed by isolation on next day. *Salmonella* was isolated from all the suspected outbreaks of fowl typhoid. For isolation, mainly heart blood, liver, spleen and bile was taken. From bile, 100% isolation was recorded followed by heart blood, liver and spleen. *Salmonella* was also isolated from the ova of a dead parent bird. On MLA, smooth pin point transparent (non lactose fermenter) colonies were seen. The single colony from MLA when streaked on BGA for selective isolation, small, smooth, dew drop like colonies with pink background were observed. Positive colonies on BGA showed agglutination with positive serum against *Salmonella* Gallinarum.

Biochemical results of confirmed positive cultures were shown in Table 1.

Table 1. Biochemical characterization of isolates

Biochemical test	*Salmonella* Gallinarum	*Salmonella* Pullorum	*Salmonella* Enteritidis	*Salmonella* Typhimurium
Motility	-	-	+	+
Urea	-	-	-	-
H_2S	-	-	+	+
Indole	-	-	-	-
MR	V	V	+	+
VP	-	-	-	-
Citrate	V	V	+	+
Glucose	A	A	Ag	Ag
Dulcitol	+	-	+	+
Salicin	-	-	-	-
Sucrose	-	-	-	-
Galactonate	-	-	-	-
Malonate	-	-	-	-
ONPG	-	-	-	-
Lysine	+	+	+	+
Maltose	+	V	+	+
Sorbitol	-	-	+	+
lactose	-	-	-	-
Arabinose	V	+	+	+

V, Variable; -, negative; +, positive; A, acid production; Ag, acid and gas production.

Our results were in accordance with the traditionally accepted characteristics as well as with the findings of Mdegela *et al*. (2000). However some variability in the biochemical characters as reported by Shah *et al.* (2001) was seen in our isolates too.

Serotyping of 88 positive cultures of *Salmonella* was carried out in CRI Kasauli. Different serotypes of *Salmonella* namely *Salmonella* Gallinarum, *Salmonella* Pullorum which are host adopted along with important zoonotic serotypes *Salmonella* Enteritidis and *Salmonella* Typhimurium were reported (Table 2).

Table 2. Relative occurrence of different serotypes of *Salmonella* from poultry during 2008

Serotype	Number isolated	Relative occurrence (%)	Antigenic structure
Salmonella Gallinarum	53	57.60	9,12:-:-
Salmonella Pullorum	16	17.39	9,12:-:-
Salmonella Enteritidis	13	14.13	9,12:g,m:-
Salmonella Typhimurium	06	6.52	4,12:1:1,2
Total	88		

Table 3. Antibiogram pattern of different isolates of *Salmonella*

Serotype	Total isolate	C		AK		G		CE		CQ	
		S (%)	R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)
Salmonella Gallinarum	53	36(67.9)	17(32.1)	18(33.9)	35(66.1)	31(58.4)	22(41.6)	07(13.2)	46(86.8)	5(9.4)	48(90.5)
Salmonella Pullorum	16	06(37.5)	10(62.5)	07(43.8)	09(56.2)	10(62.5)	06(37.5)	01(6.3)	15(93.7)	01(6.3)	15(93.7)
Salmonella Enteritidis	13	07(53.9)	06(46.1)	03(23)	10(77)	06(46.1)	07(53.9)	01(7.7)	12(92.3)	01(7.7)	12(92.3)
Salmonella Typhimurium	06	02(33.4)	04(66.6)	00	06(100)	00	06(100)	00	06(100)	00	06(100)

C, chloremphenicol (30 mcg); Ak, amikacin (30 mcg); G, gentamicin (10 mcg); CE, cephotaxime (10 mcg); CQ,cephadroxil (30 mcg).

Many of these serotypes have also been reported earlier in cultures from various sources and places in India. *Salmonella* Gallinarum was found to be one of the major serovars affecting poultry in India (Verma *et al*. 2000). The present study also revealed that the prevalence of *Salmonella* Gallinarum was maximum followed by *Salmonella* Pullorum, *Salmonella* Enteritidis and *Salmonella* Typhimurium. Prakash *et al.*(2005) observed that serovars of *S.* Gallinarum and *S.* Enteritidis were predominant. Second most common serotype encountered in the present study was *Salmonella* pullorum and this is the first published report, showing its emergence in Northern India. However there are few reports in other parts of India and the world. Isolation of serotypes like *Salmonella* Typhimurium and *Salmonella* Enteritidis in the present study signifies the importance of other serovars in poultry and is an indication of close association between man and poultry during the farm operations. Similar observations were made by Rahman *et al*. (2002) and Prakash *et al*. (2005).

Scanning electron microscopy

The scanning electron microscopy results helped in making more accurate phenotypic classification of the *Salmonella* serovars based upon the size, shapes and the presence of the flagella. Scanning electron microscopy of *Salmonella* serovars revealed rod shaped morphology (Fig.3a-d). *Salmonella* Gallinarum and *Salmonella* Pullorum being non motile were seen without flagella in different fields. Moreover bacterial rods of *Salmonella* Gallinarum were longer (1.0–2 × 1.5μm) than *Salmonella* Pullorum (0.3–0.5 × 1.25 μm).

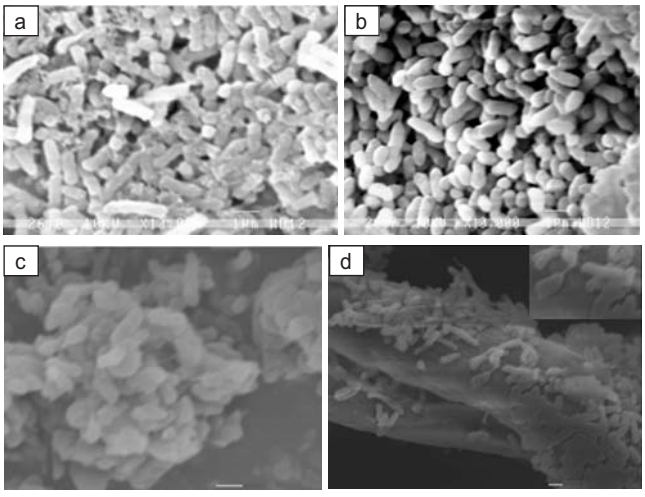

Fig 3 (a) *S.* Gallinarum at 10000× . (b) *S.* Pullorum at 10000×. (c) *S.* Typhimurium at 9000× showing peritrichous flagella. (d) *S.* enteritidis at 5000× showing monotrichous flagella.

In the present study, peri-trichous flagella were detected in *Salmonella* Typhiumurium, i.e flagella projecting from most of the bacterial surface making them tumbly swimmers. Similar observations were recorded by Prakash *et al*. (2005).While *Salmonella* Enteritidis had single flagella projecting from one end of the rod i.e. monotrichous flagella proving the fact that both are motile organisms. *Salmonella* Gallinarum and *Salmonella* Pullorum being non motile were seen without flagella suggesting their non motile type of nature.

Antibiogram pattern

In recent years, antibiotic resistance in *Salmonella* has assumed alarming proportions worldwide. Monitoring drug resistance pattern among the isolates, not only gives vital clues to the clinician regarding therapeutic regime to be adopted against individual cases, but is also an important tool to devise a comprehensive chemoprophylactic and chemotherapeutic drug schedule on flock basis within a geographical area. *Salmonella* serotypes confirmed after serotyping were tested for antibiotic sensitivity and zone of inhibition was recorded using antibiotic zone scale and interpreted as resistant and sensitive based on values given in zone size interpretive chart (Table 3). Six antibiotics, namely chloramphenicol, amikacin, gentamicin, cephotaxime and cephadroxil, used in the present study were selected on the basis of their extensive use. Majority of the isolates showed drug resistant pattern.

Salmonella Gallinarum and *Salmonella* Enteritidis isolates showed maximum sensitivity to chloramphenicol. Gentamicin was found to be the second most effective antibiotic. Similar observations were reported by Bhattacharya *et al*. (2001). However Muniyellappa *et al*. (2003) found sensitivity of organisms against chloramphenicol but most of the isolates were resistant to gentamicin because of its regular use in poultry industry in

that particular area. *Salmonella* pullorum showed maximum sensitivity to gentamicin (62.5%),where as *Salmonella* Typhimurium isolates were 100% resistant to all antibiotics used in the study except chloremphenicol which showed sensitivity for 33.4% isolates. Our findings were in agreement with the results reported by Rahman *et al*. (2002) and Murugkar *et al*. (2005). Most of the isolates in present study showed resistance against cephotaxime and cephadroxil, possibly because of their indiscriminate use for preventive and curative purposes as also found by Bhattacharya *et al*. (2001). Resistance to these higher generation antibiotics may be due to the fact that a particular antibiotic is effective at the time of introduction and later on becomes ineffective once it is used inadvertently for a long duration and/or in sub optimal doses. Hence, it is appropriate to select an antibiotic based on antibiogram pattern rather than earlier reports of their effectiveness, because no single antibiotic would be effective at all times (Prakash *et al.* 2005).By analyzing the antibiogram pattern of all the isolates, it was found that all the isolates were resistant to more than one antimicrobials used, indicating the prevalence of multiple drug resistance which substantiates the findings of Shivhare *et al*. (2000), Shah *et al*. (2001), Bhattacharya *et al*. (2001), Carraminana *et al*. (2004) and Siemon *et al.* (2007).

Study concluded that the appearance of substantial multiple resistance in *Salmonella* isolates suggests the need for more prudent use of antibiotics in treatment and prophylaxis. Antibiotic selection should be based upon in-vitro testing. So need of hour is to control the infection at parent level and to study its molecular epidemiology to know the changes in the organism at the genotypic level.

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