

Prevalence of *Salmonella* spp. on exterior and interior of fertile eggs from vaccinated breeding hens of different breeds

Abstract

Aim: To study prevalence and distribution of *Salmonella* serotypes on exterior and interior of fertile eggs from four different breeds of poultry and to assess the antibiotic drug resistant pattern of isolates.

Material and Methods: Egg samples were taken on random basis from exterior and interior of eggs from four different breeds of poultry viz. Vanaraja, Gramapriya, Black Rock and Kalinga Brown reared at govt. Poultry Farm, Durg (CG). Samples were processed for isolation of *Salmonella* spp. using selective enrichment media (Rappaport Vassiliadis Medium) and differential media (MacConkey Lactose Agar) and selective media (Brilliant Green Agar). Isolates were identified by standard biochemical tests and characterized by serotyping using antisera specific for O and H antigen. Each of the isolates was tested for their sensitivity to different antibiotics using disc diffusion method.

Results: Breed wise cultural prevalence of *Salmonella* serotypes was highest (41%) in Vanaraja and Gramapriya followed by 33% in Kalinga Brown and 25% in BlackRock. Out of 17 total isolates, 16 were recorded as *S. Enteritidis* and one as *S. Typhimurium*. Antibiogram of most prevalent *Salmonella* isolates on the farm revealed that Ciprofloxacin and Enrofloxacin was most effective (100% sensitive) followed by Chloramphenicol (64% sensitive) while all isolates showed resistance to Amoxicillin and Cephalixin.

Conclusion: Higher prevalence of *Salmonella* serotypes was recorded in fertile eggs of poultry. *Salmonella* serotypes included *S. Enteritidis* and *S. Typhimurium*.

Keywords: prevalence, *salmonella*, fertile eggs, poultry breed, antibiogram

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Introduction

Salmonellosis is one of the most common infections of poultry and other host species including human. It causes food borne diseases in humans when eggs may get contaminated by horizontal or vertical contamination.¹ *Salmonella* have been known to cause illnesses for more than 100 years and are Gram negative bacilli belonging to the Family *Enterobacteriaceae*. Like most *Enterobacteriaceae* they are motile, nonspore forming and facultative anaerobes that reduce nitrates to nitrites, ferment glucose, and are oxidase negative.² Among the serovars, *Salmonella enterica* subspecies *enterica* serovar *Enteritidis* and *Salmonella enterica* subspecies *enterica* serovar *Typhimurium* can bind to isthmal secretions and can be incorporated into egg during formation. Eggshells and associated membranes have been found to be a frequent site of *S. Enteritidis* and *S. Typhimurium* contamination after naturally and experimentally induced *Salmonella* infections.³ Egg contents may also become contaminated with *Salmonella* from soiling of the shell with faeces of infected hens, either by direct penetration of the shell or on breaking the eggs. In light of diverse modes of transmission, possibility of 'superinfection', complex mechanism of pathogenesis and antigenic diversity, the study about cultural prevalence which includes isolation, identification and characterization of *Salmonella* in the flock carries greater significance. Generally advanced poultry production practice

promises full protection by immunization which is cleverly designed by combination of breeder hen vaccination and active immunization of chicks at appropriate age during early life. Despite of moderate to high percentage of seropositivity due to efficient active immune response to *Salmonella* vaccine administered to the breeding hens, cultural prevalence of *Salmonella* have been recorded from eggs.^{4,5} In recent years, the rapid emergence of multidrug resistance bacteria caused by the wide application of antibiotics in clinical practices has caused great difficulties in medical treatment.⁶ There are many recent literatures on antibiogram of *Salmonella* isolates⁷⁻⁹ showing different resistance patterns of *Salmonella* spp. to different antibiotics and importance of it in emergence of multiple drug resistance. To foresight and formulate strategic measures for the control and proper treatment of vertically transmitted disease like salmonellosis in a commercial poultry flock, the present study was planned to investigate the rate of re-occurrence and comparative prevalence of salmonellosis in actively immunized breeding hens of four breeds viz. Vanaraja, Gramapriya, Black Rock and Kalinga brown.

Material and methods

Present study included 48 laying hens (40-60weeks of age) from twelve each of four breeds of poultry viz. Vanaraja, Gramapriya, Black Rock and Kalinga Brown reared under optimum management condition at Government Poultry Farm, Durg (Chhattisgarh). These

hens were vaccinated with *Salmonella* polyvalent vaccine inactivated (Venky's). Twelve eggs from each flock were selected during normal hatching and samples from exterior and interior of eggs were collected on random basis.

Isolation of *Salmonella* from eggs

The standard methodology¹⁰ was used for isolation and identification of *Salmonella* from exterior surface as well as inner contents of eggs. Egg swabs were taken from the surface of each egg by dipping a sterile swab in normal saline and then swabbing over the surface of egg. The swabs were then dipped in a separate tube containing 5 ml RVM.

Each of the eggs was broken and a drop of egg yolk pipette out to inoculate in RVM for selective enrichment. After incubation for 24hr, tubes showing turbidity were separated and loop full of growth was further streaked on MLA, incubated at 37°C for 24hr. Lactose fermenting (pink colonies) and non lactose fermenting (colourless colonies) organisms were identified. The *Salmonella* suspected non lactose fermenting colonies were picked up and streaked on BGA. The plates were incubated at 37°C for 24hr. The isolated single colony appeared pink on BGA. A pure culture on nutrient agar slants was preserved at 4°C till further processing.

Identification and characterization of *Salmonella* isolates

All the isolates suspected as *Salmonella* spp. were further identified on the basis of Gram staining, motility and biochemical characters. Gram staining and motility test was performed on each isolate and observations were considered for presumptive identification for *Salmonella* spp. Each of the isolates was further identified on the basis of series of biochemical tests viz. Indole production, Methyl red, Voges-proskauer and Citrate utilization.¹¹ Observations about the set of biochemical tests; Citrate utilization test and pattern of growth in Triple sugar iron agar slant (TSI) were further considered for final identification of *Salmonella* up to the species level. On Simmon's citrate agar each inoculum from pure culture was streaked and subsequent after 72hours incubation at 37°C, these slants were observed for change of colour from green to blue. Similarly isolates were inoculated on the TSI agar slants by stab culture method into the butt and incubated aerobically at 37°C for 24hr. TSI isolates were serotyped by slide agglutination test using commercially available antisera specific for O and H antigens of *S. Typhimurium* and *S. Enteritidis*.

Antibiogram of isolates

Each of the isolates was tested for their sensitivity to different antibiotics as per method described by Bauer et al.¹² Commercially available antibiotics discs (Himedia, Laboratories Limited, Mumbai) were used to test sensitivity of isolates against the antibiotics. Antibiotics discs included Amoxicillin, Chloramphenicol, Chlortetracycline, Ciprofloxacin, Cephalexin, Doxycycline, Enrofloxacin, Furazolidone, and Sulphadiazine. From the pure culture a loop full of culture was inoculated into 5.0ml of nutrient broth and incubated at 37°C for 18hr. The inoculum was spread over the Muller and Hinton agar plates with the help of sterile swab and left for 20min in incubator for absorption of culture. The antibiotic discs were placed at equal distance with the help of pointed forceps without touching to surface of agar. After overnight incubation, diameter (mm) of zone of inhibition (if any) was measured using Hi antibiotics Zone scale (Himedia) and susceptibility of isolates to antibiotic was recorded.

Results

Isolation and identification of *Salmonella* spp.

Based on primary culture or enrichment in RVM (Figure 1) followed by differentiation on MLA (Figure 2) and specific growth characters on BGA (Figure 3), isolates were presumptively identified as *Salmonella* spp. On Gram's staining isolates showed presence of small Gram negative *coccobacillus* (Figure 4); were motile (Figure 5) and found positive for methyl red and citrate utilization test (Figure 6) however showed negative result with indole production and Voges-proskauer. Based on cultural characteristics and biochemical properties, isolates were confirmed as *Salmonella* spp. On TSI, One isolates showed black precipitates (more H₂S production) throughout media with less alkaline reaction on slant (indicative of *S. Typhimurium*), however 16 isolates (suspected for *S. Enteritidis*) produced black precipitate over slant surface only with red slant and yellow butt (Figure 7). Using antiserum specific for O and H antigens, 16 isolates were identified as *S. Enteritidis* and one as *S. Typhimurium*.

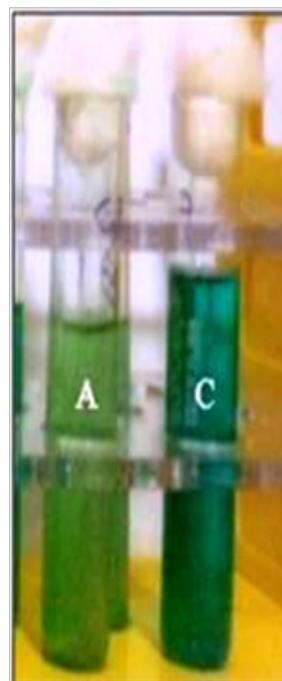


Figure 1 RVM showing turbidity
A, RVM positive; C, Control



Figure 2 Growth of *Salmonella* on BGA
A, yellow colonies on BGA; B, pink colonies on BGA (*Salmonella*)



Figure 3 Lactose fermenting and lactose non fermenting colonies on MLA
A, Lactose non fermenting colonies (*Salmonella*); B, lactose fermenting colonies

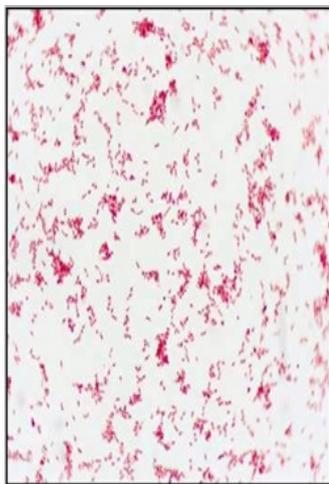


Figure 4 Gram staining showing *Salmonella*.

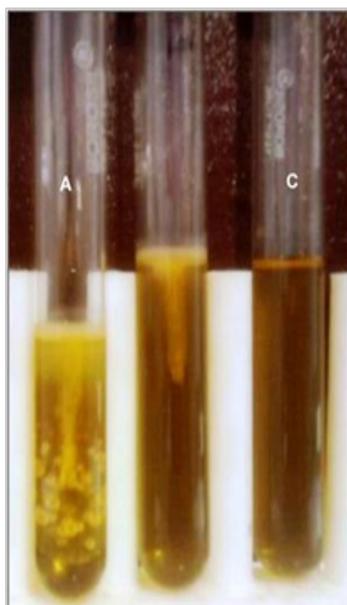


Figure 5 Motility of *Salmonella* in Edward's media
A, Motility of *Salmonella*; C, Control

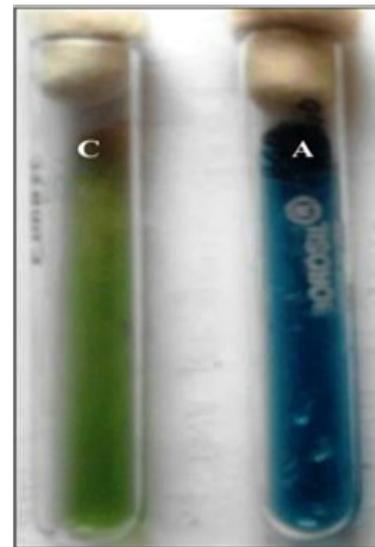


Figure 6 *Salmonella* on Simmon's Citrate Agar
A, Citrate positive; C, Control

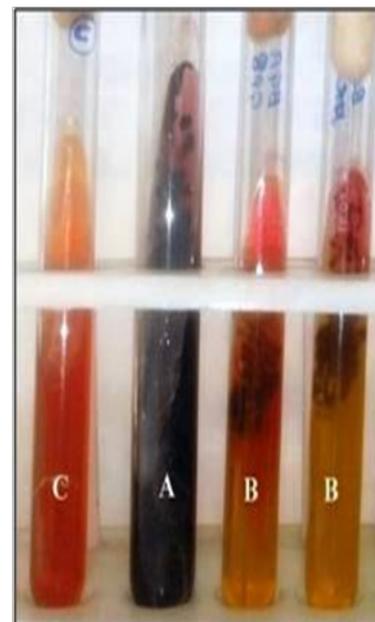


Figure 7 *Salmonella* on TSI agar
A, *S. typhimurium*; B, *S. Enteritidis*; C, control

Prevalence of *Salmonella* serotypes

35.4% (17 out of 48) egg samples were found positive for *Salmonella* spp. Exterior and interior of the eggs showed 14.5 and 20.8% cultural prevalence of *Salmonella* spp., respectively. Of total isolates (35.4%), 33.3% included *S. Enteritidis* and 2.08% belongs to *S. Typhimurium*. Prevalence rate was higher in Vanaraja and Gramapriya breed (41.6%) followed by Kalinga Brown (33.3) and Black Rock (25%). Breed wise and serotype wise distribution of *Salmonella* on exterior and interior of eggs are depicted in Table 1 & 2.

Antibiogram profile of *Salmonella* isolates

In the present study 100 % isolates were found highly sensitive

to Ciprofloxacin and Enrofloxacin (Figure 8) followed by Chloramphenicol (64%). All isolates of *Salmonella* were resistant to Amoxicillin and Cephalixin followed by Doxycycline and Furazolidone (88.23%), Sulphadiazine (76.4%) and Chlortetracycline (64%).

Table 1 Distribution of *Salmonella* serotype on exterior of eggs

Breed	No. of samples tested	No. of samples positive for <i>Salmonella</i>		
		<i>Salmonella</i> Enteritidis	<i>Salmonella</i> Typhimurium	Total positive (percent)
Vanaraja	12	1	-	1(8.3)
Gramapriya	12	2	1	3(25)
Black Rock	12	3	-	3(25)
Kalinga Brown	12	-	-	-
Total	48	6	1	7(14.5)

Table 2 Distribution of *Salmonella* serotype in interior of eggs

Breed	No. of samples tested	No. of samples positive for <i>Salmonella</i>		
		<i>Salmonella</i> Enteritidis	<i>Salmonella</i> Typhimurium	Total positive (percent)
Vanaraja	12	4	-	4(33.3)
Gramapriya	12	2	-	2(16.6)
Black Rock	12	-	-	-
Kalinga Brown	12	4	-	4(33.3)
Total	48	10	-	10(20.8)

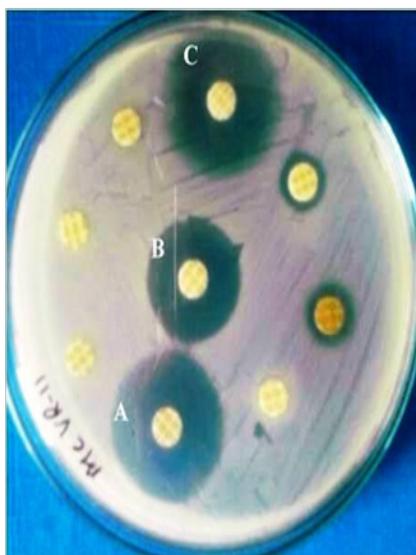


Figure 8 Pattern showing antibiogram of *Salmonella* isolates A, ciprofloxacin; B, enrofloxacin; C, chloramphenicol

Discussion

Isolation of *Salmonella* from eggs possesses great importance as they may lead to spread of *Salmonella* in hatchery. Breeder and broiler hatcheries were highly contaminated with salmonellae because many eggs coming from a naturally infected flock and even

a single *Salmonella* positive egg can increase the contamination.¹³ Eggs inoculated with high numbers of *S. Typhimurium* still hatch and upon hatch, the *Salmonella* is widely disseminated through the hatching cabinet due to fan-forced air and most (>80%) of the chicks hatched in trays both above and below inoculated eggs were positive for an antibiotic-resistant marker strain of *S. Typhimurium*.¹⁴ In another study, Cason et al.¹⁵ showed that eggs carrying *Salmonella* on the exterior or in shell membranes could lead to contamination of the chick at the pipping of the eggshell.¹⁵ Thus even single *Salmonella* infected egg is of great importance in contaminating the whole flock. Eggshells and associated membranes have been found to be a frequent site of Enteritidis and Typhimurium contamination after naturally and experimentally induced *Salmonella* infections.³

In a study, relatively higher (49.5 %) cultural prevalence of *Salmonella* from live and dead birds was reported,⁴ which might be due to the differences in environmental, managerial and geographical distribution. The percentage isolation rate of *S. Typhimurium* in present study (2.08%) is in agreement with the finding of Nagappa et al.,¹⁶ who reported 1.5% prevalence. The slight variation may be attributed to previous exposure history to *S. Typhimurium* which may have imparted immunity to the hens under study.

Prevalence of *Salmonella* serotypes in eggs from vaccinated hen could be explained. Presence or absence of antibody in the blood is not directly related with the presence of bacteria in the reproductive or digestive tract which further get transmitted to egg yolk and egg surface, respectively. Our findings are in accordance with the findings earlier reported by Ahmed et al.,⁴ who have reported 71% cultural

prevalence from cloacal swabs in seropositive birds and 59 per cent in seronegative birds.⁴ Higher cultural prevalence in seropositive birds than seronegative birds could be due to repeated exposure of hens to *Salmonella* infection along with continuous antibiotic treatment in all ages of birds. Similarly, in a report 66% of *Salmonella* was recorded in seropositive group.⁵ Present study was supported by a study, in which it was reported that only 80% hens vaccinated with *S. Enteritidis* vaccines survived through passive protection against *Salmonella enterica* serovar Enteritidis infection from maternally derived antibodies.¹⁷ Higher cultural prevalence of *Salmonella* in present study may be due to less effectiveness of solely used inactivated vaccines. The facts was supported by Dorea et al.,¹⁸ and Innoue et al.,¹⁹ who observed that passive immunity to progeny chicks has been exploited as an intervention to break the chain of infection vertically against salmonellae by vaccinating breeders with a combination of both live and killed *Salmonella* vaccines. Use of a live *Salmonella* vaccine to prevent colonization of the gut early by wild-type salmonellae and to reduce shedding of the organisms will effectively control spread and the prevalence of salmonellae in poultry.²⁰

The widespread use of antimicrobial agents in food-animal production has contributed to the decreased susceptibility of *Salmonella* to antibiotics showing 91% multidrug resistance, which can be transmitted to humans through food of animal origin²¹ and thus frequent antibiotic treatment may precede the emergence of antibiotic resistant strains of *Salmonella* spp. (Indiscriminate use of antibiotics in the field without knowing the culture sensitivity increases the risk of developing multidrug resistance *Salmonella* spp. So antibiotics must be used very judiciously to lower the development of multidrug resistance *Salmonella* serotypes.) Antibiotic sensitivity pattern in present study correlates with the observation of Kumar et al.,²² who reported Ciprofloxacin sensitive *Salmonella* isolates. The results in present study are *at par* with the results reported by Chao et al.,²³ who reported increased resistance of *Salmonella Typhimurium* strains to Doxycycline, Colistin, Sulfamethoxazole- trimethoprim and Cephalothin. Increased resistance of *Salmonella* to different antibiotics was also supported by Yan et al.,²⁴ who detected multidrug resistant genes in 177 isolates of *S. Enteritidis*.²⁴

Conclusion

Looking to the higher cultural prevalence of *Salmonella* serotypes in vaccinated breeding hens (Because of higher cultural prevalence of *Salmonella* serotypes inspite of vaccination, throws a light that the immunity provided by the vaccine is not sufficient enough which may be due to age of the birds, vaccination schedule etc, which supports the prevalence of *Salmonella* serotypes in breeding hens) further study is to be needed regarding rate of maternal transfer of immunoglobulin to progeny chick. (High level of maternal transfer of immunoglobulin to progeny chick via egg will confer resistance against *Salmonella* in both eggs and chicks) multidrug resistant *Salmonella* serotypes in present study explore the indiscriminate use of antibiotics in poultry flock. (Frequent usage of antibiotics in the poultry flock may have lead to development of multidrug resistance *Salmonella* spp.) A range of interventions should be implemented in addition to vaccination to control salmonellosis including biosecurity, pest and rodent control, judicious use of antibiotics and competitive exclusion with probiotics. (Because of presence of multidrug resistance *Salmonella* serotypes in present study in spite of vaccination, needs more measures to check the occurrence of Salmonellosis in Poultry flock which can be done

by increasing biosecurity measures, controlled and judicious use of antibiotics etc).

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Conflict of interest

Author declares that there is no conflict of interest.

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