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ORIGINAL ARTICLE

Prevalence and antibiotic resistance of Salmonella from the eggs of commercial samples

Harsha HT[1], Reshmi R[1], Rinoy Varghese[1], Divya PS[2], Mujeeb Rahiman KM[2], Mohamed Hatha AA[2]

[1]*School of Environmental Sciences, Mahatma Gandhi University, Kottayam, Kerala, India*

[2]*Dept. Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Cochin, Kerala, India*

ABSTRACT

Objectives: In India egg is being promoted as complete food by the National Egg Co-cordination Committee (NECC) and being included in the free mid-day meal provided to school student

s in rural areas. The objective of the present study is to determine the prevalence and antibiotic resistance of Salmonella on the shell and in the contents of eggs of commercial samples from the retail outlets of Kottayam, South India.

Materials and Methods: Six hundred samples of eggs (150 each from commercial layer hens, non-commercial layer hens, duck and Japanese quail) were analysed for the prevalence of Salmonella as per United States Food and Drug Administration Bacteriological analytical manual and the confirmed and serotyped strains were analysed for antibiotic resistance by Kirby-Bauer disk diffusion method.

Results: Five different serotypes such as *S.weltevreden*, *S.worthington*, *S.dublin*, *S.bareilly* and *S.typhimurium* were encountered in the eggs of commercial layer hens, while backyard raised layen hen eggs had only *S.bareilly* and *S.dublin*. Japanese quail eggs showed prevalence of *S.worthington*, *S.typhimurium* and *S.bareilly*. *S.enteritidis* was encountered only in the duck eggs where the only other serotype was *S.infantis*. Conclusion: *S.enteritidis* prevalence was relatively less when compared to reports from other countries. More than 50% of duck eggs had Salmonella contamination in the contents which pose threat to unwary consumers. Resistance to tetracycline, oxytetracycline and nalidixic acid were relatively high among Salmonella isolates from eggs of commercial layer hens. *J Microbiol Infect Dis 2011; 1(3):93-100*

Key words: Salmonella, hens' egg, duck egg, quail egg, antibiotic resistance

Ticari yumurta örneklerinden Salmonella prevalansı ve antibiyotik direnci

ÖZET

Amaç: Hindistan'da yumurta, Milli Yumurta Koordinasyon Komitesi (NECC) tarafından tam besleyici gıda olarak teşvik edilmeye başlandı ve kırsal alandaki okullarda öğrencilere ücretsiz sağlanan gün ortası öğününe dahil edildi. Bu çalışmanın amacı, Güney Hindistan Kottayam'da perakende satış noktalarından temin edilen ticari yumurtaların kabuk ve içeriğinde Salmonella prevalansını ve antibiyotik direncini araştırmaktır.

Gereç ve yöntem: Altıyüz yumurta örneğinde (150'şer adet ticari yumurta tavuğu, ticari olmayan yumurta tavuğu, ördek ve Japon bıldırcınından) Salmonella prevalansı ve antibiyotik direnci Birleşik Devletler Gıda ve İlaç Yönetiminin Bakteriyolojik analiz el kitabına göre araştırıldı ve doğrulanan ve tiplendirilen suşlarda Kirby-Bauer disk difüzyon yöntemiyle antibiyotik direnci çalışıldı.

Bulgular: Ticari yumurta tavuğu yumurtalarından *S.weltevreden*, *S.worthington*, *S.dublin*, *S.bareilly* ve *S.typhimurium* olarak beş farklı serotip izole edilirken bahçede yetiştirilen yumurta tavuğu yumurtalarından sadece *S.bareilly* ve *S.dublin* serotipleri izole edildi. Japon bıldırcını yumurtalarından *S.worthington*, *S.typhimurium* ve *S.bareilly* izole edildi. Ördek yumurtalarında ise *S.enteritidis* ve *S.infantis* ile karşılaşıldı.

Sonuç: Başka ülkelerden bildirilenlerle karşılaştırıldığında *S.enteritidis* sıklığı göreceli olarak daha azdır. Ördek yumurtalarının % 50'den fazlası içeriklerinin Salmonella ile kontamine olmalarından dolayı tüketiciler için tehdit oluşturmaktadır. Ticari yumurta tavukların yumurtalarından izole edilen Salmonella şuşlarında tetrasiklin, oksitetrasiklin ve nalidiksik asi-de karşı direnç göreceli olarak yüksekti.

Anahtar kelimeler: Salmonella, tavuk yumurtası, ördek yumurtası, bıldırcın yumurtası, antibiyotik direnci

Correspondence: A.A. Mohamed Hatha, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Cochin -682 016, Kerala, India. Email: mohamedhatha@gmail.com, Fax: 0484 -2368120.

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INTRODUCTION

Food-borne diseases caused by Salmonella serotypes occur at high frequency in industrialized nations and developing countries and represent an important public health problem worldwide.[1-2] Most of the Salmonella infections in humans' results from the ingestion of contaminated poultry[3] and these infections are associated with the consumption of raw eggs and foods containing raw eggs. In India isolation of salmonellae has been periodically reported from poultry, man, foods of animal origin and also from other sources.[4-6] Backyard hens can also be infected through contact with wild animals, domestic mammals and commercial poultry that are carriers of salmonellae and consequently may play a role in the transmission of the organism to other animals and humans.[7] In addition, *S.enteritidis* infection in chickens is often silent-with no evident morbidity or mortality among infected chickens-so there may not be any outward indication of *S.enteritidis* infection among chickens in farm flocks or in the eggs they produce.[8]

The administration of antimicrobial agents in chickens creates selection pressure that favors the survival of antibiotic resistant pathogens. Multidrug-resistant phenotypes have been increasingly described among Salmonella species worldwide.[9] Prevalence of antimicrobial resistant Salmonella in broiler chicken and foods of animal origin has been reported from India.[10-11] Most other reports of antibiotic resistance among Salmonellae in India are from clinical isolates such as *S.typhi*.[12-13]

In India consumption of the eggs have gone up considerably during the last decade due to the efforts of National Egg Co-ordination Committee (NECC), Govt. of India, which promotes egg as complete food and encourage consumption of at least an egg a day. Many State governments in India are also supplying free eggs to school children as a part of free mid-day meal scheme, especially in rural schools. However, the eggs are considered as potential food vehicles for Salmonella contamination, systematic studies to track the prevalence of Salmonella among them are rarely undertaken in India. Hence the present study has been taken up to generate baseline data on prevalence and distribution of various Salmonella serotypes in different types of eggs such as eggs from commercial and backyard hens, duck and Japanese Quail, that are being consumed in the study area and to evaluate the risk associated with them by carrying out antibiotic resistance analysis.

MATERIALS AND METHODS

Sampling and processing for Salmonella

Eggs were collected at random from different retail outlets near medical college, Kottayam, Kerala, India. Total of 600 eggs (150 each for commercial layer hen, backyard raised layer hen, duck and Japanese quail (locally known as Kada) were analyzed for the presence of Salmonella. Unwashed eggs were collected individually in sterile polythene bags and transported to the laboratory. Samples were subjected to bacteriological examination within 1 hour after sampling.

Swab technique was used to sample the egg surface of the intact eggs. The swabs were directly inoculated into 10ml buffered peptone water for pre-enrichment, in screw capped bottles and incubated at 37°C for 24 hrs. In order to collect the egg content, the eggshell was aseptically broken and contents of each egg was transferred into 225 ml of buffered peptone water, homogenized and incubated at 37°C for 24 hrs. Samples were processed as per Bacteriological Analytical Manual (BAM) of United States Food and Drug Administration (USFDA).14 One ml of pre-enriched culture was selectively enriched in 10 ml of tetrathionate broth (TTB) at 37°C for 24 hours and streaked on to selective media such as xylose lysine deoxycholate (XLD) agar and hektoen enteric agar (HEA) plates and incubated at 37°C for 24 to 48 hours.

Plates were observed after 24 hours incubation for typical Salmonella like colonies on XLD and HEA, whenever present typical Salmonella like colonies were picked up and maintained on TSA slants at room temperature for further characterization. The stored cultures were subjected to primary biochemical screening, which includes reactions in Triple Sugar Iron Agar (TSI) and Lysine Iron Agar (LIA), indole production in Tryptone Broth and urea splitting ability on Christiansen's Urea Agar. Cultures matching the reactions of Salmonella in preliminary biochemical screening were further subjected to carbohydrate fermentation tests with lactose, sucrose, dulcitol and salicin. All the bacteriological media used in

the study were purchased from Hi-Media Laboratories Ltd., Mumbai, India. The cultures matching the biochemical reactions of Salmonella were further confirmed by slide agglutination test using polyvalent O sera (Wellcome Laboratories, Dartford, England) and serotyped at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, Himachal Pradesh.

Antibiotic sensitivity testing

Antibiotic susceptibility tests were carried out by the disk diffusion method15. Briefly, the Salmonella isolates were enriched in nutrient broth for 16-18 hours at 37°C before swabbing on to surface dried plates of sterile Mueller Hinton Agar (MHA). After 15 minutes of pre-diffusion time, antibiotic disks were placed on the seeded agar surface, sufficiently separated from each other so as to avoid overlapping of inhibition zones. The plates were then incubated at 37°C for 24 hours, diameter of inhibition zones were recorded and compared with the Kirby Bauer chart for the interpretation of results.

The antibiotics used and their concentration was as follows: amikacin (30 mcg); ampicillin (10 mcg), chloramphenicol (30 mcg), ciprofloxacin (5 mcg), gentamycin (10 mcg), kanamycin (30 mcg), streptomycin (10 mcg), tetracycline (30 mcg), vancomycin (30 mcg), novobiocin (30 mcg), nalidixic acid (30 mcg) and oxytetracycline (30 mcg).

Statistical analysis

The results were subjected to Two-Factor ANOVA without replication by using SPSS (Version: 11; SPSS Inc.1995).

RESULTS

Prevalence of Salmonella in Eggs of Poultry, Duck and Japanese quail

Salmonella contamination in commercial layer hen eggs, backyard hen eggs, duck and Japanese quail eggs collected from the retail outlets from Kottayam has been studied in the present investigation. A total of 600 eggs (150 each for different types) were analyzed for the presence of Salmonella. The prevalence of Salmonella on egg content ranged from 1.33% in commercial layer hen to 51.33% in duck eggs (Table 1). None of the egg contents of Japanese quail tested positive for Salmonella. Except in duck eggs shell surface had relatively higher levels of Salmonella contamination in case of the eggs from commercial layer hens, backyard layer hens and Japanese quail when compared with their egg contents. The prevalence of Salmonella on eggshell ranged from 4% to 20.66% (Table 1). Shell contamination was maximum on commercial layer hen eggshells when compared with other types of eggs. Eggshell contamination with Salmonella was less than 10 percent in the eggs of backyard layer hen, Japanese quail and duck.

Relative prevalence of Salmonella on the eggshell and in the contents of different types of eggs indicates that, in the case of duck eggs, egg content had considerably higher levels of contamination when compared with eggshell. Statistical analysis of prevalence of Salmonella in the egg contents and on the eggshell of different types of eggs revealed that there was no significant difference ($P>0.05$) in the prevalence of Salmonella in the egg contents and on the eggshell of different types of egg.

Distribution of various Salmonella serotypes in different types of eggs

Table 2 indicates the relative incidences of various Salmonella serotypes in different types of eggs. Seven different serotypes such as *S.worthington*, *S.weltevreden*, *S.typhimurium*, *S.bareilly*, *S.dublin*, *S.enteritidis*, and *S.infantis* were isolated from different types of eggs analyzed in the present study. While commercial layer hen eggs showed diverse Salmonella serotypes, the duck egg content had only two serotypes such as *S.infantis* and *S.interitidis*. The presence of *S.dublin* was observed both in commercial layer hen eggs and backyard raised layer hen eggs. *S.bareilly* was isolated from the eggs of commercial layer hen, non-commercial layer hen and Japanese quail. *S.typhimurium* was present in eggs of commercial layer hen and Japanese quail. The presence of *S.worthington* was recorded both in the egg of commercial layer hen and Japanese quail but the presence of *S.weltevreden* observed only in commercial layer hen eggs.

Table 1. Prevalence of Salmonella on the eggshell and in the contents of different types of eggs

Type of Egg	No. of Samples analysed	Incidence of Salmonella, %	
		Eggshell	Egg contents
Commercial Layer Hen	150	20.66 (31)	1.33 (2)
Back yard raised Layer Hen	150	4 (6)	2 (3)
Duck	150	6 (9)	51.33 (77)
Japanese Quail	150	6 (9)	0 (0)

Table 2. Prevalence of different Salmonella serotypes in different types of eggs

Type of egg	*Salmonella* serotype	Antigenic pattern	Incidence, %
Commercial layer hen	*S.worthington*	1,13,23:z:1,w	50
	S.weltevreden	3,10:r:z6	4.17
	S.typhimurium	4,5:I:1,2	8.33
	S.bareilly	6,7:y:1,5:-	20.83
	S.dublin	9,12:g,p:-	16.67
Backyard raised layer hen	*S.bareilly*	6,7:y:1,5:-	77.78
	S.dublin	9,12:g,p:-	22.22
Duck	*S.enteritidis*	9,12,g,m:-	53.33
	S.infantis	6,7:r:1.5	46.66
Japanese Quail	*S.worthington*	1,13,23:z:1,w	28.57
	S.typhimurium	4,5:I:1,2	14.29
	S.bareilly	6,7:y:1,5:-	57.14

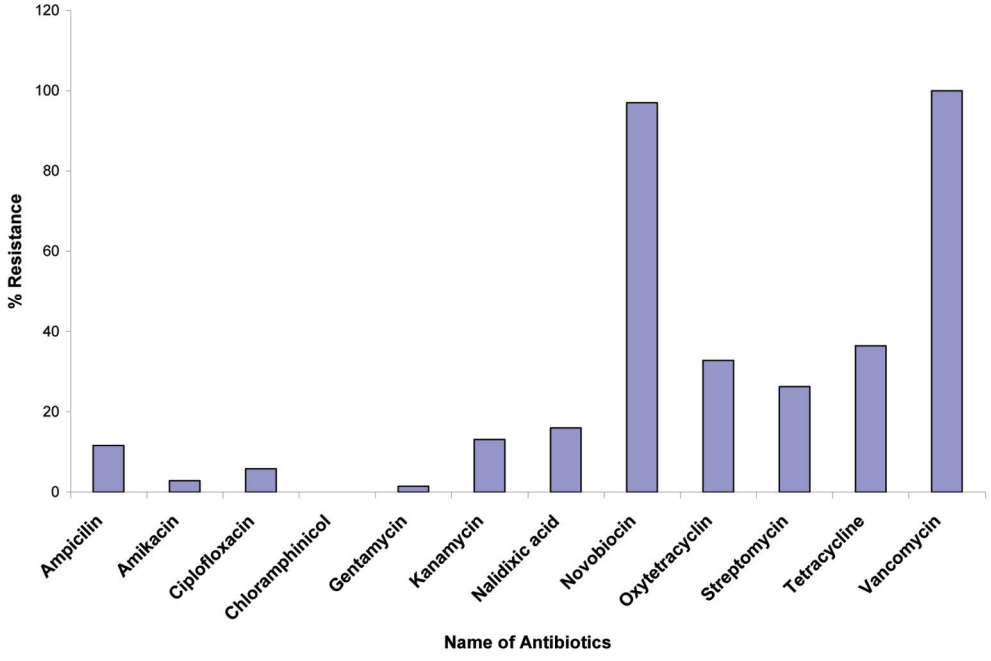

Figure 1. Overall percentage of antibiotic resistance among the Salmonella isolates from eggs

Table 3. Variation in the percentage of antibiotic resistance among the Salmonella isolates from various types of eggs

Antibiotic	Percentage of resistance among *Salmonella* isolates from the eggs of			
	Commercial Layer Hen	Backyard Layer Hen	Duck	Japanese Quail
Ampicilin	12.12	83.3	1.16	33.3
Amikacin	3.03	33.3	1.16	0.00
Ciprofloxacin	6.06	16.6	0.00	0.00
Chloramphenicol	0.00	0.00	2.32	22.2
Gentamycin	0.00	16.6	0.00	0.00
Kanamycin	27.27	50	1.16	44.4
Nalidixic acid	45.45	33.3	1.16	11.11
Novobiocin	100.00	100	100.0	100
Oxytetracycline	60.60	33.3	22.09	33.3
Streptomycin	27.27	50	26.74	33.3
Tetracycline	63.63	50	24.41	33.3
Vancomycin	93.93	100	100	100

Antibiotic resistance of Salmonella strains from eggs

A total of 137 strains of Salmonella (33 from commercial layer hen eggs, 9 from backyard layer hen eggs, 9 from Japanese quail eggs and 86 from duck eggs) were tested for their susceptibility to 12 different antibiotics. Overall resistance among Salmonella from eggs is given in Figure 1. While all the isolates were resistant to vancomycin, none of them was resistant to chloramphenicol. Novobiocin resistance was also very high. More than 25% of the isolates were resistant to oxytetracyclin, streptomycin and tetracycline. Relatively low level of resistance was observed against other antibiotics.

Variation in the antibiotic resistance among Salmonella isolates from different types of eggs is presented in Table 3. While resistance to vancomycin was uniformly high among Salmonella isolates from different types of eggs, it was found to vary in case of resistance to other antibiotics. Resistance to tetracycline, oxytetracycline and nalidixic acid were higher among Salmonella isolates from eggs of commercial layer hens. Drug resistance was relatively less among Salmonella isolates from the eggs of duck and Japanese quail.

DISCUSSION

Salmonellosis is still a major food borne disease in human and the significance of Salmonella spp. as causes of human and animal disease has increased in the recent years.[16] Modern practices in the poultry industry are even now very favorable to the maintenance and dissemination of Salmonella serotypes.[17] The present investigation revealed the prevalence of diverse serotypes of Salmonella in commercial layer hen eggs when compared to other types of eggs such as eggs from layer hens maintained at backyards, duck and Japanese quail. Another highlight of the investigation was high degree of Salmonella contamination in the contents of duck egg, with two specific serotypes such as *S.enteritidis* and *S.infantis*.

In the case of commercial layer hen eggs around 20% of eggshells were contaminated with Salmonella. The incidence levels of Salmonella in eggshell reported earlier were variable. Singh, Yadava, Singh and Bharti6 reported relatively lower level of incidence in the chicken eggs collected from poultry farms and marketing channels in North India. While Perales & Audicana.[18] reported around 1% Salmonella contamination in Spain prevalence levels were reported to be varying from zero to 7% in the United Kingdom.[19] The prevalence level in the present investigation is higher than these observations.

The Salmonella contamination in the commercial layer hen seem to be horizontal as most of the Salmonella positive samples were from eggshell, which is likely to be cross contamination from faeces or from the cage environment. Since the shell contamination of egg is a case of horizontal transmission, prompt removal of poultry waste and disinfection can greatly reduce this. This is further strengthened by our observation that shell contamination in backyard layer hen eggs was much lower as these hens are maintained at the backyard at a much lower concentration. Shell contamination was negligible on the eggs of Japanese quail eggs also. Hunaeu-Salaun et al [20] studied the risk factors of Salmonella enterica subsp. Enterica contamination in layer flocks and reported that the contamination is more in caged birds than in on-floor flocs.

The results of the present study highlights high degree of Salmonella contamination in the duck egg contents with *S.enteritidis* and *S.infantis*. This is indicative of trans-ovarian route of contamination by *S.enteritidis* and *S.infantis*. It is reported that the contamination of egg contents with *S.enteritidis* is predominantly the result of infection of the reproductive tissue rather than passage through the shell after lay.[21] These researchers also observed that there was no association between shell contamination and the presence of *S.enteritidis* of egg content laid by naturally infected hens. *S.enteritidis* is a serovar that can infect and cause disease in a broad range of hosts including poultry and number of mammalian species.[22] *S.enteritidis* is the most common serotype reported in human cases of salmonellosis in the European Union and the second most common serotype (behind *S.typhimurium*) reported in the United States.[1,23] Like most Salmonella serotypes, *S.enteritidis* has a variety of virulence factors that contribute to its pathogenicity. There was no Salmonella contamination in the egg content of Japanese quail eggs.

Five different Salmonella serotypes such as *S.worthington*, *S.weltevreden*, *S.typhimurium*, *S.bareilly* and *S.dublin* were encountered in the eggs of commercial layer hens. While the diversity of Salmonella serotypes were much less in other types of eggs. This is indicating the commercial layer farms are good source of diverse Salmonella serotypes. The result also clearly indicate that *S.worthington* was the most frequently isolated serovar on the eggshell along with other serovars, such as *S.bareilly*, *S.typhimurium*, and *S.dublin*. In our results other serovars of Salmonella spp., isolated from the eggshells were not recovered in the egg contents. This suggests that not all the eggs with shell contamination had their contents contaminated. The contamination recorded in eggs content was only by *S.enteritidis* and *S.infantis*. Considerable prevalence of *S.infantis* have been reported from poultry products, poultry and poultry environment.[24]

Salmonella contamination, especially that with *S.enteritidis* in the egg contents of commercial and backyard layer hens were found to be negligible. *S.enteritidis* associated salmonellosis has been reported as a pandemic involving several conditions in Europe and USA in the early 90's [25] and initiatives to track the presence of *S.enteritidis* in eggs/poultry from different parts of the world has come in place. Since food production in a globalised environment is mostly centralized and the chances of outbreak from a common source could likely arise, one of the objectives of the present investigation was to generate data on *S.enteritidis* contamination in egg for comparison with existing reports of *S.enteritidis* in egg. The results clearly revealed that *S.enteritidis* is yet to establish as a predominant serotype in hens' eggs in our country.

However, *S.enteritidis* contaminations in duck eggs seem to be significant. Since the ducks in our region are usually maintained in aquatic environments with considerable degree of microbial pollution, the possibility of access of pathogen such as *S.enteritidis* from such environments to ducks is quite high. The results also indicate that *S.enteritidis* is capable of doing transovarian contamination in ducks. However specific studies are required to prove this point. Garrod and McILroy[26] reported hospital outbreak of enteritis due to duck eggs. Duck egg is preferred over hens' egg among the villagers of the study area and most households along the coastal belt maintain ducks at home which are usually allowed to feed in the nearby field and adjacent canals close to their home. There is also a practice of eating raw eggs among the villagers as they consider it more nutritious. Hence the high prevalence of Salmonella in duck eggs poses definite threat to unwary consumers in the study area.

Another popular food item among the locals is Japanese Quail eggs, which is cheaper

and considered highly nutritious. While none of the quail eggs had Salmonella contamination in their contents, 3 different serotypes such as *S.typhimurium*, *S.worthington* and *S.bareilly* were isolated from the shell surface. Sander et al.,[27] reported dynamics of Salmonella contamination in commercial quail operations. However, reports from India are not available for comparison.

The antibiogram of the Salmonella isolates encountered in the present study revealed that most of the strains had acquired resistance to more than 4-5 antibiotics. Multidrug resistant Salmonella in chicken eggs from poultry farms of North India has been reported recently6. While more than 90% of the Salmonella isolates were resistant to novobiocin and vancomycin, resistance to other antibiotics were relatively low. Overall resistance to antibiotics was slightly higher when compared to the resistance levels among egg borne salmonellae from China,[28] while comparable to those from Korea.[29] However the resistance levels encountered in current study were much lower when compared to reports from Iran.[30]

More than 30% of the Salmonella isolates were resistant to oxytetracyline and tetracycline. Resistance to these two antibiotics was much lower than those reported from Taiwan.[31] Tetracycline has been used to treat day-old chickens, to control infection by Salmonella, E. coli and mycoplasma, which might have resulted in the emergence of tetracycline resistant Salmonella in the layer and broiler flocks.[32] This is evident in the results from our study, where we observed relatively higher levels of oxytetracycline and tetracycline resistance among the Salmonella isolates from commercial layer hen eggs and non-commercial layer hen eggs.

Ampicillin resistance among the Salmonella strains encountered in the present study was lower than those reported by Suresh, Srinivasan, Hatha and Lskshmanaperumalsamy.[11] Ciprofloxacin is a fluoroquinolone antibiotic that is increasingly and successfully used for the treatment of septicemia in humans and ciprofloxacin resistance in human and veterinary Salmonella isolates has occasionally been found.[33] An interesting observation made in the present study is elevated levels of ciprofloxacin resistance among Salmonella isolates from backyard layer hen eggs. These hens are maintained at the backyard of household, where they might get exposure to strains from humans, where ciprofloxacin is widely used in treatment.

Gyles[9] reported that Salmonella, the frequencies and patterns of antimicrobial resistance vary depending on time, region, serovar, the particular farm, layers versus broilers, and the antimicrobial agent. Also, wide spread use of antibiotics in animal production systems is contributing significantly to the increased antibiotic resistance among pathogenic bacteria of animal origin.[34] Salmonella contamination in the layer flocks is the major cause of spread of this organism and strategies such as competitive exclusion, phage therapy and production of specific pathogen are being practiced in many countries to contain this problem. Major challenge to researchers world over is containment of emerging strains of Salmonella, as highlighted by the emergence of non-typhi Salmonellae when *S.typhi* strains were targeted. Though *S.enteritidis* association with transovarian contamination is well proven, other strains are also showing such capabilities, which throws in new challenges. Though the results of present study highlights the considerable degree of Salmonella contamination of duck egg contents by two serotypes such as *S.enteritidis* and *S.infantis*, focused research on this aspect is required to establish transovarian contamination of duck eggs with *S.infantis*.[5]

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