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## ISOLATION AND IDENTIFICATION OF *ESCHERICHIA COLI* AND *SALMONELLA* FROM POULTRY FEED AND LITTER AND THEIR ANTIMICROBIAL RESISTANCE PROFILE

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**ABSTRACT:** Avian salmonellosis is a large group of acute or chronic diseases of birds caused by different species of the genus *Salmonella*. It is a problem of economic concern for all phases of the poultry industry, from production to marketing. The main aim of this study is to investigate the incidence of *Salmonella* species in the feed and environment of open-system poultry farms. A total of 46 samples were taken from six poultry farms, layers and broilers. The samples include poultry feed from feeders (23 samples) and litter (23 samples). Isolation of *Salmonella* was carried out in a selective classical medium (DCA) after enrichment in Selenite-F broth. Four *Salmonella* isolates represent 5% of total samples were recovered; three isolates (75%) from litter samples and one isolate (25%) recovered from water samples; no *Salmonellae* were recovered from feed samples. All isolates were identified at the species level using cultural characteristics and biochemical reactions. An antimicrobial sensitivity test for the four *Salmonella* isolates was carried out. Each isolate was tested to 10 different antibiotics using Mueller and Hinton Agar Medium. All isolates were found sensitive to chloramphenicol, ceftizoxime, and amikacin and resistant to gentamycin, tetracycline, ampicillin/ sulbactam, and piperacillin/ tazobactam.

**INTRODUCTION:** Nowadays, poultry industry is the fastest-growing agricultural sector. India is one of the world's largest producers of eggs and poultry meat, producing 34 billion eggs and about 600,000 tons of poultry meat. Over the years, the poultry industry in India has contributed approximately 100 billion rupees to the gross national product <sup>1</sup>. Analysts estimate that the Indian poultry industry has been growing at a much faster pace.

The advancement of the poultry industry in India is interrupted by a number of constraints, of which the major one is the outbreak of disease <sup>2</sup>. The major etiological agents are microorganisms, parasites, management causes, environmental causes, and deficiency of minerals and vitamins.

The poultry diseases are more commonly caused by *Escherichia coli*, *Salmonella*, *Listeria monocytogenes*, *Campylobacter* species, fungus, etc <sup>3</sup>. Although more than 2300 serotypes of *Salmonella* have been identified, only about 10% of these serotypes have been isolated from poultry <sup>4</sup>. The distribution of *Salmonella* serotypes from poultry sources varies geographically and changes over time. Poultry feeds and litter contaminated with bacteria pathogenic to humans can contribute

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to human foodborne illness through the feed-poultry-food-human chain<sup>5</sup>. The production of poultry feeds requires microbiological safety regulations to avoid microbial contamination of the product. According to the report from the US Department of Agriculture (USDA) National Veterinary Service Laboratory, the most commonly identified species in chickens in the United States were *S. heidelberg*, *S. enteritidis*, *S. hadar*, *S. montevideo*, *S. Kentucky* and *S. typhimurium*<sup>6</sup>. The significance of poultry as a reservoir for human salmonellosis can be illustrated by considering the species commonly isolated from humans. The pathogens discharged from the chicken contaminate the litter, feed, water, and nearby birds. Chicken is arguably the most popular poultry meat in India. Its share in total meat consumption is 28%, as against 14% ten years ago<sup>5</sup>. One of the leading causes of foodborne infections in India is *Salmonella* from consuming poultry products.

Members of the family Enterobacteriaceae are gram-negative, non-spore-forming rods. Some of them are human and animal pathogens that cause intestinal infections and food poisoning. The genera of pathogenic importance in poultry include *Salmonella* and *Escherichia*<sup>7</sup>. Avian salmonellosis is an inclusive term designating a group of acute or chronic diseases of fowl caused by different species of the genus *Salmonella*, including *S. pullorum* (pullorum disease), *S. gallinarum* (fowl typhoid), *S. arizonae* (arizonae infection), *S. enteritidis*, and others (paratyphoid infection)<sup>8</sup>.

Paratyphoid infections are economically among the most important bacterial diseases in the hatching industry and result in high death losses among all types of young poultry. In addition, the occurrence of this disease in valuable breeding stocks is extremely costly. Also, fertility, hatchability, and egg production may be seriously impaired<sup>9, 10</sup>. Adult birds infected with paratyphoid organisms generally show no outward symptoms; however, they may serve as intestinal carriers of the infection over long periods of time and serve as the chief source of paratyphoid infections in most species of poultry<sup>11</sup>. Fecal contamination of egg shells with paratyphoid organisms during the process of laying or from contaminated nests, floors, or incubators after laying is of foremost importance in the spread of the disease. Also, the disease may be transmitted

directly to young birds from older fowl that are chronic intestinal carriers of the infection but exhibit no visible symptoms<sup>11</sup>.

Evidence has been presented that poultry feeds may be a common and very important source of paratyphoid organisms. The level of *Salmonella* contamination in poultry feeds is normally low; however, it has been shown that even one organism per 15 grams of feed can produce infection<sup>12</sup>. Salmonellosis in poultry resulted in continuous increase in public health problems<sup>13</sup>.

Contamination of poultry meat with *Salmonella* was investigated by many scientists in Sudan as well as in many other countries. In Sudan, it has been possible to isolate 21 *Salmonella enteritidis* from embryonated eggs<sup>14</sup>. Another study highlighted the occurrence of *Salmonella* in poultry carcasses in Khartoum state; 23 serotypes were identified, and most of them were *S. monas* and *S. amek*<sup>15</sup>.

Next to *Salmonella* strains, the Avian pathogenic *E. coli* (APEC) causes localized or systemic infection outside the avian gut, which is indicated as extra intestinal Pathogenic *E. coli* (ExPEC). The infection caused by ExPEC is termed colibacillosis which is an infectious disease characterized by acute fatal septicemia or sub-acute fibrinous pericarditis, airsacculitis, salpingitis, and peritonitis affect broiler chickens aged 4–6 weeks<sup>16</sup>. Colibacillosis is a common bacterial disease of economic importance in poultry through decreasing the infected birds' productivity, increasing mortality, condemnation of infected carcasses at slaughter, and prophylaxis and treatment cost and is reported worldwide where humans get infections through this environment<sup>17, 18</sup>.

APEC is considered a primary or secondary pathogen in poultry. Strains that carry virulence genes (adhesin, invasins, toxins, resistance to host serum, iron acquisition systems, temperature-sensitive hemagglutinin, and K1 capsule) have all been shown to contribute to APEC pathogenesis<sup>19</sup>,<sup>20</sup> and could induce colibacillosis without previous immune suppression factors such as stress or concurrent infections<sup>21</sup>. The control and prevention of bacterial diseases in food animals are achieved by the application of antimicrobials during periods

of high risk of infectious bacterial diseases, as prophylactic treatment, and as growth promoters<sup>22</sup>. Bacterial antimicrobial resistance develops naturally over time; the unprecedented increase of antimicrobial-resistant organisms is linked to the massive use of antimicrobial agents for disease control and prevention in human and animal medicine<sup>18</sup>.

Several forces play a role in the spread of antimicrobial resistant bacteria, including the presence of carrier animals moving between animal herds and vector action<sup>23</sup>.

The study will develop awareness about the bio-security measures in poultry farm, and help preventing contamination of bird with *E. coli* and *Salmonella* from feed and litter and transmission to humans.

## MATERIALS AND METHODS:

**Type of Study:** Cross-sectional study

**Place of Study:** Cuddalore, Tamil Nadu

**Duration of Study:** Two months

Institutional Ethical Committee (IEC) clearance was obtained (TSRMMCH&RC/ME-1/2021 – IEC No: 002 dated 05.08.2021).

**Collection of Samples:** Litter and feed samples of 10g each were randomly collected from five commercial broiler farms in Cuddalore district, Tamil Nadu. We planned to collect a total of 50 samples from the broiler farms, with at least 5 litter and 5 feed samples from each farm. But certain ups and downs happened **Table 1**.

Samples were collected aseptically and transferred immediately into a sterile plastic container with a cap. The samples were then brought to the Microbiology laboratory within 6 hours for processing. Overall, the specimens were taken from poultry farms (layers and broilers) in the Cuddalore district of Tamil Nadu. Samples (feed and litter) were selected from five poultry farms during the period of two months between August and September 2021.

**TABLE 1: ORIGIN, TYPE AND NUMBER OF SAMPLES COLLECTED**

Source	Number and types of sample examined	
	Feed	Litter
Farm 1	4	5
Farm 2	3	4
Farm 3	5	6
Farm 4	6	4
Farm 5	5	4
Total	23	23

**Inoculation in Enrichment Medium:** Each sample collected in a sterile plastic container was diluted with sterile phosphate buffered saline (PBS) and kept for 1 hour. Then one (1 ml) of sample was incubated in nine (9ml) of nutrient broth for enrichment and incubated overnight at 37°C. These samples were inoculated onto Nutrient Agar (NA), *Salmonella-Shigella* Agar (SS Agar), Brilliant Green Agar (BGA), Eosin methylene blue (EMB), and MacConkey agar for bacterial isolation.

**Purification and Identification:** Non-lactose fermenter colonies were purified by repeated subculture on nutrient agar. Pure isolates were stored on nutrient agar slopes at a low temperature of 4°C. By using standard laboratory procedures based on colony morphology, Gram's staining, and biochemical tests, all the isolates were identified.

**Antimicrobial Susceptibility Testing:** As per the CLSI guidelines, all the isolated strains of *Salmonella* species and *E. coli* were tested for antimicrobial resistance using the Kirby-Bauer disc diffusion method. The sensitivity of species of *Salmonella* and *Escherichia coli* isolates to a number of antimicrobial agents was determined by the standard disk diffusion method. Each isolate was tested against 10 different antimicrobial agents used for Gram-negative bacteria **Table 2**.

Colonies from each isolate were emulsified in nutrient broth and shaken thoroughly to obtain a homogenous suspension of the test culture. The Mueller Hinton agar (MHA) plates were then flooded with the bacterial suspension and tipped in different directions to cover the whole surface of the MHA plate. Excess fluid was aspirated, and the

plates were left for 30 minutes to dry at 37°C. The antimicrobial disks were placed on the agar medium using sterile forceps. The plates were then incubated at 37°C and examined after 24 hours for

zones of inhibition, which were measured in mm. The isolates were described as resistant, intermediate, and sensitive to different antimicrobial agents.

**TABLE 2: ANTIBIOTIC DISCS USED FOR SENSITIVITY ANALYSIS**

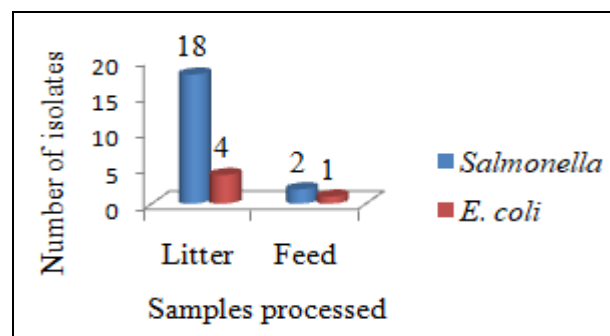
Antibiotics	Concentration	Zone of inhibition (diameter in mm)		
		Resistant	Intermediate	Sensitive
Amikacin	30mcg	14 or less	15-16	17 or more
Ampicillin/ Sulbactam	20mcg	11 or less	12-14	15 or more
Cefotaxime	30mcg	14 or less	15-22	23 or more
Ceftizoxime	30mcg	14 or less	15-19	20 or more
Chloromphenicol	30mcg	12 or less	13-17	18 or more
Ciprofloxacin	5mcg	15 or less	16-20	21 or more
Co-trimoxazole	25mcg	10 or less	11-15	16 or more
Gentamycin	10mcg	13 or less	14-15	16 or more
Piperacillin/ Tazobactam	100/10mcg	17 or less	18-20	21 or more
Tetracycline	30mcg	14 or less	15-18	19 or more

The collected data were analyzed for the prevalence of the isolates and their anti-microbial resistance profile using simple descriptive statistics such as mean, percentages, and histograms.

**RESULTS AND DISCUSSION:** A total of 46 samples were subjected to bacteriological examination. The bacterial isolation was possible with 8 litter samples (34.8%) and 2 feed samples (8.7%) **Table 3**. Among them, 25 isolates were possible; thereby, Gram-negative *Salmonella* species were identified with 18 and 2 among litter and feed samples, respectively. Among *E. coli* isolates, 4 and 1 isolates were possible while processing the litter and feed, respectively **Fig. 1**.

**TABLE 3: POSSIBLE ISOLATIONS OF THE SAMPLES**

Samples	Number of samples possible to isolation	Percentages
Litter (n=23)	8	34.8
Feed (n=23)	2	8.7
Total (n=46)	10	21.7



**FIG. 1: DETAILS OF ISOLATES**

The *Salmonella* isolates from samples gave lactose fermenter colonies (pink colonies) in DCA, and *E. coli* showed metallic sheen colonies in EMB medium. All samples that gave positive results for the appropriate biochemical test and the species of *Salmonella* differentiated. The detailed descriptions of the bacterial isolates from broiler farm litters and feed are depicted in **Table 4**. The descriptive analysis of the bacterial isolates with their species of concern is impregnated in **Table 5**.

**TABLE 4: ISOLATED BACTERIA FROM DIFFERENT SAMPLES**

Bacterial isolates	Farm 1		Farm 2		Farm 3		Farm 4		Farm 5		Total
	L	F	L	F	L	F	L	F	L	F	
<i>Salmonella typhi</i>	1	-	1	-	2	-	2	-	-	-	6
<i>S. paratyphi A</i>	-	-	-	-	1	-	-	-	1	-	2
<i>S. paratyphi B</i>	-	-	-	-	1	-	1	-	1	-	3
<i>S. typhimurium</i>	-	-	1	-	-	-	1	-	2	-	4
<i>S. enteritidis</i>	1	-	-	-	1	-	-	1	1	1	5
<i>E. coli</i>	1	-	1	-	2	-	-	1	-	-	5
Total	3	-	3	-	7	-	4	2	5	1	25

[L = Litter; F = Feed]

The sensitivity patterns of the selected *Salmonella* and *E. coli* isolates were analyzed, and sensitivity test of the four *Salmonella* isolates against 10

antibacterial agents was carried out. All isolates were found sensitive to chloramphenicol, ceftizoxime, amikacin and resistant to gentamycin,

tetracycline, ampicillin/ sulbactam and piperacillin/ tazobactam.

**TABLE 5: SPECIES CONCERN OF VARIOUS BACTERIAL ISOLATES (N=46)**

Bacterial isolates	Number of isolates
<i>Salmonella typhi</i>	6 (13)
<i>S. paratyphi A</i>	2 (4.3)
<i>S. paratyphi B</i>	3 (6.5)
<i>S. typhimurium</i>	4 (8.7)
<i>S. enteritidis</i>	5 (10.9)
<i>E. coli</i>	5 (10.9)

[Figure in parenthesis denote percentages]

**TABLE 6: SENSITIVITY VERSES RESISTANT PATTERN OF THE SALMONELLA ISOLATES**

Isolate	Antibiotics and resistant characters									
	AK	AS	CF	CI	CH	CP	CT	GT	TZP	TE
S.t	S	R	R	S	S	R	IN	R	R	R
S.pA	S	R	IN	S	S	R	IN	R	R	R
S.pB	S	R	IN	S	S	R	IN	R	R	R
S.tm	S	R	R	S	S	R	R	R	R	R
S.e	S	R	R	S	S	IN	R	R	R	R

[AK – Amikacin, AS – Ampicillin/ Sulbactam, CF – Cefotaxime, CI – Ceftizoxime, CH – Chloromphenical, CP – Ciprofloxacin, CT – Co-trimaxazole, GT – Gentamycin, TZP – Piperacillin/ Tazobactam, TE – tetracycline; S.t – *Salmonella typhi*, S.pA – *Salmonella paratyphi A*, S.pB – *Salmonella paratyphi B*, S.tm – *Salmonella typhimurium*, S.e – *Salmonella enteritis*; S-sensitive, R-resistant, IN-intermediate]

*E. coli* was isolated from five (5) samples (four from litter and one from feed) related to gastrointestinal infections due to contaminated feed or water. Antibiotic sensitivity of *E. coli* isolated from various pathological samples revealed a low

All isolates were found sensitive to co- trimoxazole except one isolate of *S. enteritidis* found resistant; two isolates of *S. enteritidis* were resistant to cefotaxime, while the other two isolate were moderately sensitive to this agent; all isolates were found resistant to ciprofloxacin except one isolate of *S. enteritidis*. The detailed sensitivity pattern of five selected isolates of *Salmonella* species, one from each species group was tabulated **Table 6**.

sensitivity to ampicillin and tetracycline and also significant decreases in sensitivity to TMP-SMX, amoxicillin, and amoxicillin-clavulanic acid **Table 7**.

**TABLE 7: ANTIBIOTIC SUSCEPTIBILITY OF E. COLI STRAIN ISOLATED**

Isolate	Antibiotics and resistant characters									
	AK	AS	CF	CI	CH	CP	CT	GT	TZP	TE
E.c 1	S	R	R	S	S	S	S	S	R	R
E.c 2	R	R	R	S	S	S	S	S	S	R
E.c 3	S	S	R	S	S	S	R	R	S	S
E.c 4	S	R	R	S	R	S	R	R	S	R
E.c 5	S	R	R	S	S	S	R	S	R	R

[AK – Amikacin, AS – Ampicillin/ Sulbactam, CF – Cefotaxime, CI – Ceftizoxime, CH – Chloromphenical, CP – Ciprofloxacin, CT – Co-trimaxazole, GT – Gentamycin, TZP – Piperacillin/ Tazobactam, TE – tetracycline; E.c – *Escherichia coli*; S-sensitive, R-resistant]

Salmonellosis is a major public health concern and continues to have a serious economic impact on the poultry industry in all countries<sup>7, 24</sup>. With the great expansion of the poultry industry, the wide-spread occurrence of avian salmonellosis has ranked it as one of the most important egg-borne bacterial diseases of poultry. The present study was conducted to investigate the contamination of poultry feed and the poultry environment with *Salmonellae* in traditional poultry farms in Cuddalore district of Tamil Nadu. Other studies highlighted that *Salmonellae* were isolated together

with other bacterial genera as *Serratia*, *Proteus*, *Citrobacter*, *Enterobacter*, *Yersinia*, *Kluyvera* and *Hafnia*. But in this study, we concentrated on *Salmonella* and *Escherichia coli*. Although all collected samples in the study were cultured first in the selenite-F broth, gram- negative bacteria other than *Salmonella* were isolated. This can be explained by the fact that selenite F broth enriches the growth of *Salmonella* and *Shigella* but does not kill other enteric bacteria that under other conditions (subculture in DCA), can grow. The *Salmonella* isolation rate (5%) was comparable to

that reported in other studies. A study examined 1488 samples and isolated 58 *Salmonellae*, which comprise 3.9% of total isolates<sup>15</sup>. In another study, 610 samples from poultry in the Sudan and isolated 45 *Salmonellae* which counted for 7.4% of the total isolates were examined<sup>25</sup>. The later study showed a higher isolation rate compared to the findings of this study, and that may be due to the large difference in the number of samples collected in both studies. A study examined 102 samples from sick chickens in Khartoum state and isolated three *Salmonella*, which counted (2.9%)<sup>26</sup>.

*Salmonella* was isolated only from samples obtained from a farm of layers and from a farm of broilers. It was not isolated, however, from animal production research center farms or from a farm in another area. This finding did not indicate that *Salmonella* was not present in these areas, but might be due to the small number of collected samples. On the other hand, it confirms the presence of *Salmonella* contamination in farms from which *Salmonellae* were isolated.

The higher isolation rate was obtained from a farm of layers, despite the fact that all samples were collected from open-system farms; this can be due to poor hygiene on this farm. Among the examined samples, the highest rate of isolation was obtained from litter samples (three isolates), followed by water samples (one isolate).

This finding indicates a high shedding of *Salmonella* from the intestinal tracts of birds on this farm. *S. enteritidis* is the most important serovar in poultry flocks, and recently it has been of high occurrence worldwide<sup>27</sup>. In another study, it was highlighted that *S. enteritidis* could attach to granulose cells in the preovulatory membrane and subsequently infect the ovum during ovulation. On the other hand, *S. enteritidis* has the ability to penetrate eggs through the shell pores and cause egg contamination.

In the present study, three isolates of *S. enteritidis* were recovered, and our finding confirmed previous records<sup>14, 27</sup> that *S. enteritidis* was detected. As long as Sudan depends on importation of the chickens, it could have come with infected imported flocks. From view of public health, human salmonellosis was reported to have

increased recently in France and the United States of America due to *S. enteritidis*<sup>29</sup>. It was reported to cause food poisoning due to the consumption of under-cooked egg dishes<sup>30</sup>. Isolation of this bacterium from some farms represents a real threat to public health.

The antimicrobial sensitivity test was carried out for *Salmonella* isolates. All strains of *Salmonella* were found to be sensitive to chloramphenicol, ceftizoxime, amikacin and resistant to ampicillin/sulbactam, piperacillin/ tazobactam, tetracycline and gentamycin. Also, all isolates were found sensitive to co-trimoxazole except one isolate of *S. enteritidis*; two isolates of *S. enteritidis* were found sensitive to cefotaxime, while *S. arizonae* and the other isolate of *S. enteritidis* were moderately sensitive; *S. arizonae* and two isolates of *S. enteritidis* showed resistance to ciprofloxacin, while the other isolate of *S. enteritidis* was moderately sensitive.

Resistance to gentamycin has been reported, with 10% resistance to this agent determined from 105 *Salmonella* isolates<sup>31</sup>. Also, there was an increasing development of quinolone resistance all over the world<sup>32</sup>. Treatment failure due to a reduced susceptibility to ciprofloxacin in *Salmonella* is now well established<sup>33</sup>. In general, *Salmonella* is the most important agent implicated in outbreaks of food-borne diseases around the world<sup>34</sup>. Effective control or eradication programs for salmonellosis depend on a good management system, the identification of carrier birds, and accurate medication.

**CONCLUSION:** They were imposing strict regulations for animal biosecurity, and hygienic conditions and zoonotic diseases. Improve protection, prevention and control disease programs in poultry farms. Improve innate animal immunity as the frontline of disease prevention and control.

The emerging disease should receive maximum attention. Unify and regulate global animal and poultry movement and trade of domestic and wild animals. Assure product quality and impose new programs to prevent zoonotic disease transmission. Considering poultry laborers are frontline workers and are vitally important, not disposable, supported them with all necessary protections, such as

physical and financial health, to establish vital and cost-effective measures. This strategy included improving the educational background of workers through continuing education and training programs, improving biosecurity and hygienic measurements for poultrymen, slaughterhouses, and feed plants, and improving farm biosecurity and hygiene.

Strategically, the COVID-19 pandemic has taught us that research must continue and be reoriented to discover new vaccines for all living creatures. For farms, fast and affordable diagnostic tools and supplementary methods to prevent diseases are urgently needed. Research and development in poultry disease identification and control should not be limited to currently known diseases. It should be prospective and incorporate emerging zoonotic diseases that may require new vaccines for their control. Continuous education programs should be implemented at all levels of the poultry industry and must be renewed every three years. Implementing key measures will ensure that workers' financial stability and well-being are prioritized.

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