ISOLATION, VIRULENCE CHARACTERIZATION AND ANTIBIOTIC RESISTANCE PROFILING OF NON-TYPHOIDAL SALMONELLA ISOLATED FROM LAYER CHICKEN FARMS IN SOUTH INDIA

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ABSTRACT

Salmonellosis is an important food-borne zoonosis caused by *Non-typhoidal Salmonella* (NTS), found localized in the intestinal tract of livestock and poultry. Human beings acquire infection through the ingestion of raw or undercooked contaminated food of animal origin, mainly from consumption of chicken meat and eggs. Namakkal region of Southern India is the highly populated poultry producing region of the world. Though there are sporadic reports about the incidence of NTS in poultry no much information is available on the occurrence of NTS in this region. Hence, the present study was carried out to understand the prevalence and antibiotic sensitivity pattern of non-typhoidal Salmonellae in chickens of the Namakkal region of South India. A total of 56 boot swabs were collected from 28 commercial layer farms. Salmonella isolation and identification was carried out according to the guidelines of ISO protocols and the final confirmation was done by polymerase chain reaction (PCR) method. The antibiotic sensitivity of all the isolates was carried out using Kirby Bauer disc diffusion method. In the present study out of 56 boot swabs screened, four flocks in two rearing units of one farm was found positive for NTS by culture, biochemical tests and PCR. The antibiotic sensitivity of salmonella isolates was as follows: Levofloxacin (100 %), Enrofloxacin (100 %), Penicillin (100%), Cefotaxime (100%), Erythromycin (100%), Gentamicin (100%), Amikacin (100%), Azithromycin (50%), Chlortetracycline (9 %), Co-trimoxazole (0 %) and Amoxicillin (0 %).

Key words: Food borne zoonosis, non-typhoidal salmonellosis, chicken meat and eggs, poultry

Introduction

Salmonellosis is an important food-borne zoonosis caused by Non-typhoidal Salmonella, which is a Gram-negative bacterium that belongs to the family Enterobacteriaceae (Lamichhane *et al.,* 2024). These bacteria are usually found localized in the intestinal tract of livestock, poultry, pets, wild animals, rodents, reptiles and amphibians. Human beings acquire this infection through the ingestion of raw or undercooked contaminated food of animal origin, mainly from consumption of chicken meat and eggs (Shaji *et al.,* 2023). All Salmonella serotypes, with the exception of the typhoidal and paratyphoidal group, can cause Non-typhoidal Salmonellosis (NTS). The symptoms include diarrhea, vomiting and abdominal cramps which develop 12 to 72 hours after infection (Giannella, 1996). Among animals, poultry acts as a primary source of different NTS serotypes. The epidemiologically relevant NTS serotypes from poultry include *S. Enteritidis*, *S. Typhimurium, S. Newport* and *S. Heidelberg* (Rabsch *et al.,* 2001). It was found that *S. Enteritidis* was the most common serovar that infects humans through eggs in North America and Europe, whereas *S. Typhimurium* was the main serovar linked to external egg contamination (Chousalkar *et al.,* 2018). Poultry was responsible for 17.9% of food-borne illnesses in the United States between 1998 and 2008; among which *Salmonella ser. Typhimurium* and *Enteritidis* were responsible for 34% and 17.4% of chicken-related food-borne zoonosis, respectively (Painter *et al.,* 2018). It was estimated that the annual economic loss due to NTS originating from chicken would be 2.79 billion in USA. As the demand for ready-to-eat food products continues to increase globally, this concern is becoming more pressing (Scharff, 2020). In addition, the overuse of antibiotics for both treatment and growth promotion in large-scale chicken production is a growing issue that is contributing to the rise in antibiotic resistance in NTS strains. The irresponsible use of antibiotics can result in the selection of bacterial resistance, which could endanger public health by transferring the resistance from farm animals to people (Odey *et al.,* 2024). Namakkal region of Southern India is the highly populated and most dense poultry producing region of the world. Though there are sporadic reports about the incidence of NTS in poultry not much information is available on the occurrence of NTS in layer chicken housed under intensive production systems. Hence, the present study was carried out to screen for the prevalence and antimicrobial sensitivity pattern of non-typhoidal Salmonellae in chickens of the Namakkal region.

Materials and Methods

Study area

The study was carried out in the Namakkal region of Tamil Nadu in Southern India between April 2022 to October 2022. Namakkal is considered the egg basket of India, which has a population of 70 million layer hens distributed within a 50 square kilometer radius. The birds are reared under an intensive system with open-air tropical poultry houses.

Sample collection

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A total of 56 samples were collected from 28 commercial layer farms located in and around the Namakkal district of Tamil Nadu. The samples comprised boot swabs from different flocks in a layer farm. The samples were transported to the laboratory in ice and stored under refrigerated conditions until further processing.

Isolation and identification of *Salmonella*

Salmonella isolation and identification was carried out according to the guidelines of ISO protocol as follows; pre-enrichment of the boot swab in BHI broth (1:10) followed by aerobic incubation at 36 °C for 16–20 hours. Further, 50 µl of the pre-enriched sample was positioned discretely into three different locations on Modified Semisolid Rappaport Vassiliadis (MSRV) agar and incubated at 42 °C for 24 hours. Further, a single loop of MSRV cultured medium was taken and subsequently streaked onto Xylose Lysine Deoxycholate (XLD) and incubated overnight at 37 °C. The typical black-centred colony with a reddish zone on XLD was extracted and subsequently, sub cultured in Blood agar medium. The biochemical confirmation was done by triple sugar iron (TSI) and urease tests. Final confirmation was done by molecular detection by the polymerase chain reaction (PCR) method.

Molecular typing and confirmation

DNA extraction was carried out directly from the samples by boiling 1.5 ml aliquots of sample in BHI broth for 5 minutes and the supernatant following sedimentation at 14,000 rpm for 5 minutes were used as source of DNA. The concentration of extracted DNA was estimated using a Nanodrop spectrophotometer (Nanodrop; Pretoria, South Africa). According to Croci *et al*. (2004), genus specific PCR was performed using the primer set ST11 (5'-AGC CAA CCA TTG CTAAAT TGG CGC A-3') and ST15 (5'-GGT AGA AAT TCC CAG CGG GTA CTG -3') in a 25 µL reaction mixture containing 12.5 µL of 2x PCR Master mix (Red Ampliqon), 0.5 µL of each primer (10 pmol), 10.5 µL of nuclease free water. The DNA was diluted to give a final concentration of between 10-200 ng/ µL and 1 µL of this was used in the PCR. Amplification was carried out in an Eppendorf Master cycler Gradient (Hamburg, Germany) using the following cycling condition; 95ºC for 2 minutes, 35 cycles of 95ºC for 1 minute, 54ºC for 1 minute and 72ºC for 2 minutes and a final extension step of 72ºC for 5 minutes. PCR amplicons were separated by 1.5% agarose gel electrophoresis at 80 volts and 100 bp DNA markers were used for calibration. Further serovar specific multiplex PCR was performed to identify the *Salmonella* serovars using two different primers and details regarding primers and PCR conditions mentioned in Table 1.

Antibiogram (Kirby–Bauer method)

The antibiotic sensitivity of all the isolates was carried out using Kirby Bauer disc diffusion method. The commonly used antimicrobial discs namely Amoxicillin (Am) 30 mcg/disc, Chloramphenicol (C) 30 mcg/disc, Ciprofloxacin (Cf) 30 mcg/disc, Co-trimoxazole (Co) 25 mcg/disc, Enrofloxacin (Ex) 10 mcg/disc, Gentamicin (G) 30 mcg/disc, Norfloxacin (Nx), 10 mcg/disc and Oxytetracycline (O) 30 mcg/disc were used. The sensitivity pattern of all the *Salmonella* isolates was determined using Muller Hinton Agar plates (Himedia Pvt., Ltd., Mumbai) incubated at 37° C for 16 hours. The high sensitivity zone scale was used to quantify the isolates' zone of inhibition (Hi-Media Pvt., Ltd., Mumbai). The sensitivity/resistance pattern was established in accordance with the disc manufacturer's instructions (Hi-Media Pvt., Ltd., Mumbai).

Results and Discussion

Incidence of NTS in poultry

In the present study, four commercial layer flocks in two rearing units of one commercial layer farm out of 28 farms 1/28 (3.57%) screened was found positive for *Salmonella Typhimurium* by culture, biochemical tests and PCR (Fig. 1, 2 & 3). The affected rearing units were multi-aged and housed under a raised platform type of cage housing. The litter condition of the *Salmonella* -positive flocks was good to moderate. The mortality pattern of the affected flocks was slightly above the baseline standard (3-5 birds/10000 birds). However, no clinical signs and specific post-mortem lesions could be observed in the affected flocks. The egg production performance was below average (< 5% from the standard) and the affected flocks never attained peak production. The farm was inhabited by feral birds like mynahs and crows as well as rodents (Table 2).

Table 2: Epidemiological observations of NTS infected layer chicken flocks

1.	Age	Multi-age
2.	Litter condition	Good to moderate
3.	Mortality	Slightly above the baseline
4.	Egg production performance	Below average (< 5% from the standard)
5.	Peak egg production	Not achieved
6.	Rodent infestation	Present
7.	Ferral birds	Present

Antibiogram of *Salmonella* isolates

The antibiotic sensitivity of 4 *Salmonella* isolates was determined using a selection of the most commonly used antibiotics in human treatment. The percentage of isolates regarded as sensitive to each antibiotic was as follows: Levofloxacin (100 %), Enrofloxacin (100 %), Penicillin (100%), Cefotaxime (100%), Erythromycin (100%), Gentamicin (100%), Amikacin (100%), Azithromycin (50%), Chlortetracycline (9 %), Co-trimoxazole (0 %) and Amoxicillin (0 %). Notably, all the isolates were resistant to Co-trimoxazole, Amoxicillin, and Chlortetracycline.

Table 1: Primers of *Salmonella* serovars and PCR conditions

Pathogens	Primer Name	Primer Sequence	Product size (bp)	PCR Conditions
S. enteritidis	*SdfI F*	5'-ATT CTG CTT GGC GCT CCG GG-3'	304 bp	5 min @ 95°C 1 min @ 95°C 1 min @ 56°C 2 min @ 72°C (35 cycles) 5 min @ 72°C
	SdfI R	5'-CCG TCA ACT TTC GCG TAT TT-3'		
S. typhimurium	*Flic F*	5'-AGC CAA CCA TTG CTA AAT TGG CGC A-3'	620 bp	
	Flic R	5'-GGT AGA AAT TCC CAG CGG GTA CTG -3'		

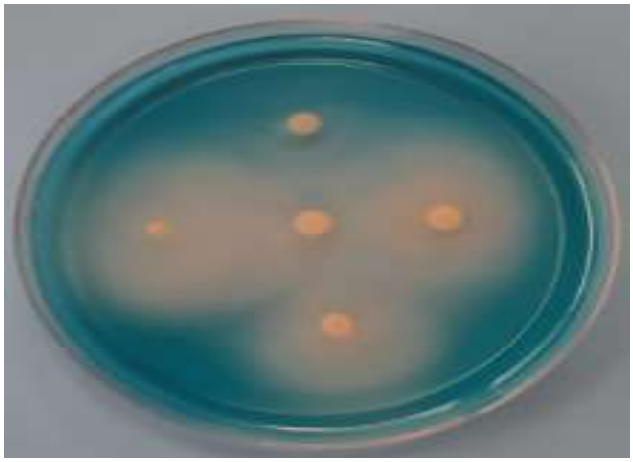

Fig. 1: NTS positive growth characterized by round zone of colonies around the inoculum in MSRV agar

Fig. 2: NTS positive growth in the affected layer flocks characterized black centred colonies in XLD agar

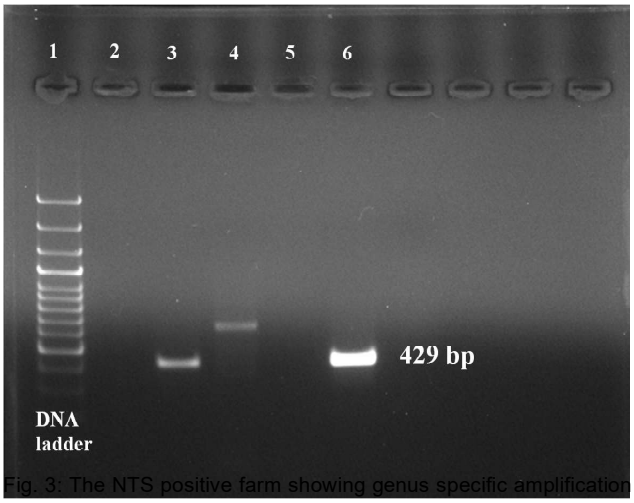

Fig. 3: The NTS positive farm showing genus specific amplification (429 bp) in PCR

The present study confirmed the prevalence of NTS in layer chicken flocks in the Namakkal region of Tamil Nadu. Typhoidal illness and nontyphoidal salmonellosis are the two common diseases associated with Salmonellosis in humans. Usually S. *enterica serovar Paratyphi* (A and B), and *S. enterica serovar Typhi* cause Typhoid fever (Schultz *et al.,* 2021). However, a number of other *Salmonella* serovars together referred to as non-typhoidal Salmonellae are responsible for non-typhoidal infections. NTS is usually associated with ingestion of contaminated meat and eggs (Ali *et al.,* 2022). The most common clinical sign of an NTS infection is self-limiting diarrhoeal illness. Furthermore, reports have indicated the prevalence of intestinal inflammation accompanied by diarrhea and extra-intestinal consequences including invasive NTS illness or bacteremia (Kumar *et al.,* 2022). The affected flocks did not show any specific clinical signs or lesions of Salmonellosis. Usually, *Salmonella* infection in poultry is characterized by a lack of obvious clinical symptoms at the farm level, however, the transmission occurs through vertical route in layers and horizontal route in broilers that usually results in contamination of poultry products with NTS that enters the human food chain, which has been identified as the primary cause of human salmonellosis (Shaji *et al.,* 2021). Hence, the presence of *Salmonella* in healthy chicken is an important risk factor for possible outbreaks of human salmonellosis (Wessels *et al.,* 2021). The results of the present study revealed that 1/28 (3.57%) of the farms screened were found positive for NTS out of 28 farms examined. The results of these findings are in accordance with the previous studies by (Saravanan *et al.,* 2015), which revealed a prevalence of 0.55 to 3.06 % of NTS in various poultry samples screened at Namakkal. However, prevalence rate is very low when compared to the prevalence of NTS in Morocco (76.7%) and Nigeria (43.6%) (Fagbamila *et al.,* 2017; Ziyate *et al.,* 2016). It denotes that layers farms in South India possesses lower risk of NTS to human. The potential risk factors found in the NTS infected flocks were the presence of rodents and wild birds. Usually, the prevalence and distribution of NTS in the farm level is influenced by various farm level and bird level risk factors including the presence of reservoirs such as rodents, reptiles, and other farm animals, contamination of the environment with farm workers, clothing, vehicles, feedstuffs, equipments, footwear, wild birds, poor hatchery hygiene, improper feed hygiene, low level of biosecurity, etc (Mkangara *et al.,* 2023). A number of variables could potentially affect the colonisation, including age, environmental and physiological stress, nutrition, *Salmonella*'s ability to survive beyond the stomach barrier, farm usage of antibiotics, chicken health, and the genetic background of the chicks (Dunkley *et al.,* 2008). Further NTS contamination of meat products is more likely when birds are slaughtered and when raw meat is handled and transported in an unsanitary manner (Chai *et al.,* 2017). *S. typhimurium* was the serovar that isolated during our investigation. This finding correlates with the earlier studies of (Saravanan *et al.,* 2015) who detected *S. typhimurium* from the various poultry clinical samples collected in and around the Namakkal region. This denotes the persistence of *S. typhimurium* in this region, and signifies the importance of identifying potential reservoirs *of S. typhimurium* for poultry in this region. The serovars that have been isolated vary depending on the continents, nations, and geographical areas. Previous reports of (Rajagopal *et al.,* 2013) suggest that *S.*

Gallinarum was the more predominant serovar in Southern India. Whereas *S. Virchow, S. Weltevreden, S. Infantis* and *S. Kentucky* were the common serovars reported in Nigeria, Philippines, Japan and the USA, respectively (Fashae *et al*., 2010, Balala *et al.,* 2009; Sandt *et al*., 2013 and Furukawa *et al.,* 2017). Environmental contamination, control systems, contamination, and sample techniques are some of the factors that contribute to this variability (Fagbamila *et al.,* 2017). The *invA* gene, codes for survival within the macrophage and also for invasion as well as the internalisation required for the invasion of deeper tissue and the subsequent development of invasive infections (Bangera *et al.,* 2018). *invA* gene was detected in all of the NTS serovars in our investigation confirming that all the NTS isolates were virulent. The antibiotic sensitivity pattern of the NTS isolates revealed that all the NTS were susceptible to most of the antibiotics used in human and veterinary practice such as Fluoroquinolones, Macrolides, Penicillins, and Cephalosporins. A higher level of resistance was found to Co-Trimoxazole (100%), Amoxicillin (100%), Chlortetracycline (91%) and Azithromycin (50%). The high resistance rate of Co-trimoxazole, Tetracycline and Amoxicillin could be due to their excessive usage of these antibiotics for long term therapeutic purposes. The antibiotic resistance pattern of our study is contrary to the previous studies of (Mir *et al.,* 2010; Fashae *et al.,* 2010) who found higher levels of Ciprofloxacin (Fluoroquinolone) resistance to NTS in Kashmir% (13.3%) and Nigeria (81%). Antibiotic resistance develops as a result of the extensive use of antibiotics in poultry practice, for preventive, therapeutic, growth-promoting, or metaphylactic purposes (Sharma *et al.,* 2024). This will lead to the mass destruction of many beneficial microflora in the gut and selective growth of resistant bacteria and their subsequent transmission to humans (Doyle *et al.,* 2006). Hence strict legislations are needed to be enforced to regulate the use and distribution of antibiotics in poultry.

Conclusions

In conclusion, the present study reveals that low prevalence of NTS in commercial layer chicken (3.57%) confirmed by cultural, biochemical and molecular techniques (PCR). The antibiotic sensitivity pattern reveals that all the isolates were sensitive to most of the antibiotics used in human and veterinary practice. However, the presence of NTS in commercial layer flocks reveals that more attention is needed to *Salmonella* control programs in Indian poultry farms since it has food safety and zoonotic implications.

Conflicts of interest

The authors declare that they have no competing interests.

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