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ANTIBIOGRAM PROFILE AND VIRULENCE GENETIC DETERMINANTS OF NON-TYPHOIDAL *Salmonella* SPECIES ISOLATED FROM THE FAECES OF CHICKENS IN OTA, OGUN STATE, NIGERIA

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ABSTRACT

Some healthy chickens harbour *Salmonella serovars*, which could potentially contaminate raw animal products during processing, and eventually be transferred to humans. This study was aimed, at determining the antibiotic resistance pattern and genetic virulence determinants that encode invasion A protein (*invA*), flagella biosynthetic protein (*fliR)* and tetrathionate reductase (*ttrA)* in *Salmonella enterica* serovars, isolated from the faeces of chickens. Two hundred faecal samples were obtained from a commercial layer poultry farm, and 25 presumptive non-typhoidal *Salmonella* strains were recovered. Antibiotic susceptibility testing (AST) was conducted by the disc diffusion method, and virulence genes were detected by polymerase chain reaction (PCR). The highest resistance among the non-typhoidal *Salmonella* in this study was to ampicillin (32%); however, a high proportion of the isolates were susceptible to chloramphenicol (96%). Out of the 25 serovars, 5 (20%) exhibited multidrug resistance to 3 or more classes of antibiotics. The *invA* gene was confirmed in all 25 (100%) *Salmonella* strains, while *fliR* and *ttrA* genes were detected in 9 (36%) and 7 (28%) of the isolates, respectively. The *Salmonella* serovars and multidrug-resistant (MDR) strains detected in the faecal samples of commercial layer flocks in this study could pose a risk to public health, as the bacteria can spread to humans via consumption. Therefore, a paradigm for more focused biosafety measures must be considered by poultry farmers.

Keywords: Antimicrobial Resistance, Virulence, Non-typhoidal *Salmonella*, Poultry, Nigeria

INTRODUCTION

Infections with *Salmonella* are one of the most common food-borne diseases, and an important global public health concern (Pui et al., 2011; Huoy et al., 2025). Numerous animals, including humans, can be infected with various *Salmonella* serotypes. The majority of human *Salmonella* infections are thought to be spread by contaminated seafood, fresh fruits, juices and vegetables, like lettuce, melon, apple, tomato and alfalfa sprouts, as well as animal-derived meats (poultry, hog and beef) (Pui et al., 2011; Mihaiu et al., 2014). According to an estimate, 80.3 million cases out of 93.8 million were food-borne cases of human *Salmonella*- related gastroenteritis, which occur every year around the world (Majowicz et al., 2010).

Several bacterial species, including *Salmonella enterica* serovars, have rotating motors called flagella that are responsible for their movement (Arnam et al., 2004). While *ttrA* is a tetrathionate reductase structural gene, which is found on *Salmonella* Pathogenicity Island (SPI), and meanwhile a variety of bacteria can employ tetrathionate as a terminal respiratory electron acceptor (Hensel et al., 1999); *invA* gene is found in the SPI region of *Salmonella*, and it is a target gene for PCR, which has been shown to be effective in detecting *Salmonella* spp., due to its distinctive DNA sequence (Abdel-Aziz, 2016). In bacterial cell membranes, *invA* gene produces proteins that are necessary for invasion into host epithelial cells (Yanestria et al., 2019).

Non-typhoidal *Salmonella* remains a serious problem globally, thus, understanding how the bacterium is distributed in poultry, is crucial for improving diseases prevention and control. In this study, we investigated the antibiotic susceptibility profile of non-typhoidal *Salmonella* serovars, isolated from faecal samples of healthy-looking chickens, and determined the presence and distribution of *invA*, *fliR* and *ttrA* genes that they may harbour.

MATERIALS AND METHODS

Samples Site

Faecal samples originating from chickens were collected between January and April 2024, from a commercial layer poultry farm along Sokoto road, Atan-Ota, Ogun State, Nigeria. The coordinates are latitudes 6.909833 (6° 54′N) and longitudes 3.2583363 (3° 15′E).

Samples Collection

A total of 200 fresh faecal samples (about 10 g of poultry droppings to make one sample) from litter at random points were collected into sterile polyethene bags, using sterile tongue depressors, and transported to the laboratory immediately at 4 ºC for processing. The study was conducted on a medium-sized commercial private farm, and consent was sought from the farm manager.

Isolation of Salmonella Strains

About 10 g of the faecal samples were transferred into 200 mL of buffered peptone water (Oxoid®, UK), and aerobically incubated for 18 - 24 h at 37 ºC. Then, 1 mL of the overnight broth culture was dispensed into 9 mL of sterile Selenite-F broth (BD Difco®, Germany), and incubated for 18 - 24 h at 37 ºC, after which the broth was streaked on *Salmonella-Shigella* (SS) agar (Oxoid®, UK), and incubated aerobically again for 18 - 24 h at 37 ºC, as described by Raufu et al. (2021). Typical *Salmonella* colonies were confirmed, based on Gram staining and biochemical assays, as previously described by Feltham and Barrow (2003).

Antibiotic Susceptibility Testing

Using the Kirby-Bauer disc diffusion method, the antibiotic susceptibility profile of the *Salmonella* isolates was assessed against a panel of eight antibiotics: ciprofloxacin (CIP, 5 µg), gentamicin (GEN, 10 µg), amoxicillin-clavulanic acid (AMC, 20 µg), cefotaxime (CTX, 5µg), chloramphenicol (CHL, 30 µg), tetracycline (TET, 30 µg), trimethoprim/sulfamethoxazole (SXT, 25 µg) and ampicillin (AMP, 10 µg), according to the recommendation of the European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2021), and the Clinical Laboratory Standard Institute guidelines (CLSI, 2020). Furthermore, the strains were classified as multidrug-resistant (MDR), if they were resistant to at least one antibiotic, in three or more classes of antibiotics (Magiorakos et al., 2012). A quality control strain (*Escherichia coli* ATCC 25922) was used in the susceptibility testing, which was conducted in duplicates.

Primer Designing

Artemis, a genome browser (Carver et al., 2012), was used to extract nucleotide sequences of *the invA* gene that encodes for a virulence-associated secretory protein, *the ttrA* gene that codes for tetrathionate reductase subunit A, and *the fliR* gene that codes for flagellar biosynthetic protein from the complete genome of *Salmonella* Typhi Ty2. Extracted sequences were saved as FASTA files and exported to the PrimerQuest tool of Integrated DNA Technologies (https://www.idtdna.com), where primers were designed. After designing the primers, a basic local alignment search tool (BLAST) run was done on each pair of primers to determine their specificity.

Molecular Detection of *invA*, *fliR* and *ttrA* Genes from Non-typhoidal *Salmonella* Isolates

DNA extraction was carried out on the overnight cultures of the 25 presumptive non-typhoidal *Salmonella*, using MiniAmp DNA extraction kit (Qiagen®, Germany), according to the manufacturer's instructions. Three genes, *invA, fliR* and *ttrA* were evaluated in this study for their potential in virulence and associated pathogenicity among non-typhoidal *Salmonella*. *E. coli, Pseudomonas aeruginosa, Providencia stuartii* and *Proteus mirabilis* were used as negative controls, while *Salmonella typhimurium* 14028 was used as a positive control.

Simplex PCR was used to detect the *invA, ttrA* and *fliR* genes, using the designed primers, given in Table 1. A 20 µL reaction mixture consisting of 10.8 µL sterile nuclease-free water, 4 µL of 5X FIREPoL master mixture (Soli Biodyn, Estonia), 0.6 µL each of forward and reverse primers, and 4 µL of DNA template was made. The PCR cycling parameters consisted of, initial denaturation at 95 ^{o}C for 5 min, followed by 35 cycles of 95 ^{o}C for 30 sec, annealing at 51 °C, 54 °C and 55 °C for *the fliR, invA* and *ttrA* genes, respectively, for 40 sec, extension at 72 ^{o}C for 1 min, and a final extension phase of 72 ^{o}C for 10 min, in an automated gradient thermal cycler (Eppendorf®, Germany). PCR products were resolved on a 1.5% agarose (Norgen Biotek Corp., Canada) gel, stained with ethidium bromide, and run in a 0.5X Tris borate EDTA (TBE) at 100 V for 60 min.

RESULTS AND DISCUSSION

Distribution of Non-typhoidal *Salmonella* Isolates and their Phenotypic Pattern of Antibiotic Resistance

A total of 25 (i.e. 12.5%) non-typhoidal *Salmonella* strains were obtained from the 200 faecal samples of healthy-looking chickens used in this study (Figure 1). Globally, non-typhoidal *Salmonella* infections in poultry pose an alarming risk to public health and food safety, since the bacterium can go undetected, contaminating both the farmlands and humans, possibly leading to gastroenteritis (Neelawala et al., 2024). The result of 12.5% non-typhoidal *Salmonella* isolated from the 200 faecal samples of healthy-looking chickens, used in this study, was within the range (4.7% to 15.9%) of the prevalence of *Salmonella* isolated from faecal samples of chickens, in earlier studies conducted in Africa (Fashae et al., 2010; Fagbamila et al., 2017; Eguale, 2018). However, Andoh and colleagues (Andoh et al., 2016) reported a higher prevalence of 44.0% flock in Ghana. Similar results of high prevalence reported in Asian nations include 46.3% of *Salmonella* spp. in poultry in central Vietnam (Lettini et al., 2016), and 41.8% *Salmonella* spp. detected in the faeces of commercial farms in Korea (Im et al., 2015). Contrariwise, non-typhoidal salmonellosis has been significantly decreasing in the United Kingdom since the implementation of vaccines in laying hens and broiler-breeder in the late 1990s (O'Brien, 2013). Furthermore, the European Union has numerous *Salmonella* control measures in place for the layer sector that are absent in developing nations (O'Brien, 2013).

Salmonella resistance to antibiotics creates a serious threat to public health and safety (Park et al., 2017; da Silva et al., 2024), and it is challenging to completely eradicate it from food animals, since they are frequently reservoir hosts for this bacterium (Eguale, 2018). From these findings, the highest resistance of non-typhoidal *Salmonella* isolates was to ampicillin (8/25; 32%), followed by tetracycline (6/25; 24%), cefotaxime (6/25; 24%), ciprofloxacin (5/25; 20%), amoxicillin-clavulanic acid (3/25; 12%), gentamicin (2/25; 8%) and chloramphenicol (1/25; 4%) (Table 2). Our findings in this study, which indicate the highest resistance among non-typhoidal *Salmonella* isolates to ampicillin, are consistent with a prior study by Bangera et al. (2019) that found 32.8% ampicillin resistance. The 32% of non-typhoidal *Salmonella* ampicillin resistance found in this study may be due to the fact that ampicillin is frequently given in water or feed to keep the chickens healthy, as well as administering treatment courses with incorrect or insufficient dosage. However, a significant proportion (96%) of the strains in this present study were sensitive to chloramphenicol. Other studies have revealed > 90% susceptibility of non-typhoidal *Salmonella* isolates to chloramphenicol (Pande et al., 2015; Bangera et al., 2019).

This study revealed that proportions of the *Salmonella* strains were susceptible to chloramphenicol (96%), gentamicin (92%), amoxicillin-clavulanic acid (88%), trimethoprim/sulfamethoxazole (88%), ciprofloxacin (80%), tetracycline (76%) and cefotaxime (76%) (Table 2). Out of the 25 *Salmonella* serovars, 5 (20%) exhibited multidrug resistance to 3 or more classes of antibiotics (Table 3). A major public health issue is the growing prevalence of MDR *Salmonella*, which is resistant not only to first-line antibiotics like ampicillin, trimethoprim/ sulfamethoxazole and chloramphenicol (Fagbamila et al., 2023), but also to critically important antibiotics, such as third-generation cephalosporins and fluoroquinolones, as listed by the World Health Organisation (WHO, 2019).

Detection of *invA*, *fliR* and *ttrA* Genes

The *invA gene* was amplified in all 25 (100%) *Salmonella* isolates analysed, as well as in the *Salmonella typhimurium* 14028 positive control. The amplification of the *invA* gene in all the isolates (100%) is consistent with the results of a previous study, which suggested that PCR amplification of the *invA* gene made a reliable, rapid diagnosis of *Salmonella* spp. (Mohammed, 2022). Invasion gene operon (*invA)* is

crucial for *Salmonella's* complete virulence, and it is considered to initiate the internalisation process, needed for the invasion into deeper tissues (Steele-Mortimer et al., 2002). According to previous reports, this gene has been found in every strain of *Salmonella* spp. (Diab et al., 2023; Salem et al., 2026). *Salmonella* genus detection by *invA* amplification has been recognised as a global standard procedure (Malorny et al., 2003). On the other hand, there was no amplification in the negative controls of *P. aeruginosa*, *E. coli*, *P. mirabilis* and *P. stuartii*, as shown in Figure 2.

Furthermore, *the fliR* gene was detected in 9 (36%) of the *Salmonella* strains, and 7 (28%) of these strains harboured the *ttrA* gene (Figures 3 and 4). In the flagellar type III secretion system (fT3SS), *fliR* is one of the three putative integral membrane proteins that make up the export gate. This is important for the virulence and motility of many major bacterial pathogens, including *Salmonella* spp. (Kuhlen et al., 2018; Hendriksen et al., 2021). The ttrBCA operon encodes tetrathionate reductase, which is essential for the infection process. TtrA and TtrB are metallo-co-factors, targeted to the periplasmic side of the membrane, by two different Tat targeting peptides. *Salmonella's* ability to use sulphur compounds like tetrathionate ($3OS\text{-}S\text{-}S\text{-}SO_3$ or $S_4O_6^{2}$) and thiosulphate ($S\text{-}SO_3$ or $S_2O_3^{2}$), as terminal electron acceptors, during anaerobic respiration is an intriguing aspect of the bacterium's physiology (Hinsley and Berkes, 2002; Stoffels et al., 2012).

Three (12%) of the *Salmonella* isolates harboured all three (i.e. *invA*, *ttrA* and *fliR*) genes, 4 (16%) of them harboured *invA* and *ttrA*, while 4 (16%) harboured both *invA* and *fliR*, as shown in Table 4. However, none of the bacteria*l strains harboured only fliR and ttrA genes* (Table 4). Based on the distribution of assayed genes in *Salmonella* serovars in this study, the *Salmonella* strains were grouped into four genotypes, including *invA*, *invA+ttrA*, *invA+fliR* and *invA+ttrA+fliR*.

CONCLUSION

Findings from this study showed that *Salmonella* serovars were detected in faecal samples of commercial layer flocks of otherwise healthy birds. *Salmonella*, particularly in poultry, can contaminate the environment, resulting in intermittent faecal shedding. Non-typhoidal *Salmonella* strains displayed multidrug resistance, indicating a potential public health threat. Therefore, in order to avoid the spread of multidrug-resistant (MDR) *Salmonella* in birds, poultry breeders should consider adopting extra hygiene, antibiotic stewardship measures and vaccination.

Authorship Contribution Statement

ATS*:* Conceptualisation, Methodology, Investigation, Formal Analysis, Resources, Writing-Original Draft Preparation, Writing-Review and Editing, Visualisation and Supervision;
GAA*:* Conceptualisation, Methodology, Investigation, Resources, Writing-Review and Editing, Visualisation and Supervision;
FOA*:* Conceptualisation, Methodology, Investigation, Writing-Review and Editing;
CJK*:* Resources, Writing-Review and Editing;
FTO*:* Resources, Writing-Review and Editing.

Data Availability Statement

The corresponding author can provide the data supporting these current study findings upon reasonable request.

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Table 1: List of PCR Primers Used for the Amplification of *invA*, *ttrA* and *fliR* Genes

Genes	Primers sequences	Products sizes (bp)
invA	F-5'-CCGATTTGAAGGCCGGTATTA-3' R-5'-GAGATCGCCAATCAGTCCTAAC-3'	587
fliR	F-5'-TCGCTACCAGCAAACGATAC-3' R-5'-GGTAAGCGGGAAACCGATA-3'	495
ttrA	F-5'-TGCTGATTATCTGGCGATTCC-3' R-5'-ACTGATGACCGCAGCTTTAC-3'	412

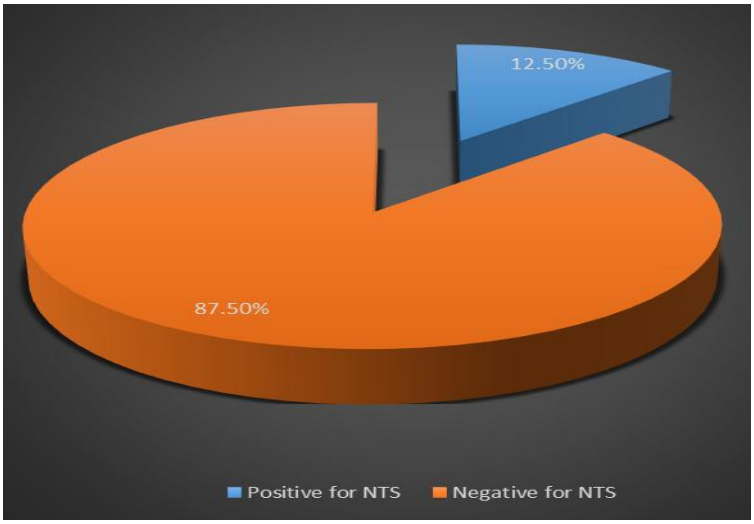

Figure 1: Isolation Rate (12.5%; 25/200) of Non-typhoidal *Salmonella* (NTS) in Faecal Samples from Chickens in Ota, Ogun State, Nigeria

Table 2: Antibiotic Susceptibility and Resistance Pattern of Non-Typhoidal *Salmonella* (NTS) Isolated from Faecal Samples of Chickens in Ota, Ogun State, Nigeria

Antibiotics	Salmonella enterica serovars susceptibility (%)	Salmonella enterica serovars resistance (%)
Gentamicin	23(92)	2(8)
Amoxicillin-Clavulanic acid	22(88)	3(12)
Tetracycline	19(76)	6(24)
Ciprofloxacin	20(80)	5(20)
Trimethoprim/Sulfamethoxazole	22(88)	3(12)
Cefotaxime	19(76)	6(24)
Ampicillin	17(68)	8(32)
Chloramphenicol	24(96)	1(4)
Gentamicin	23(92)	2(8)
Amoxicillin-Clavulanic acid	22(88)	3(12)
Tetracycline	19(76)	6(24)
Ciprofloxacin	20(80)	5(20)
Trimethoprim/Sulfamethoxazole	22(88)	3(12)
Cefotaxime	19(76)	6(24)
Ampicillin	17(68)	8(32)

Antibiotics	Salmonella enterica serovars susceptibility (%)	Salmonella enterica serovars resistance (%)
Chloramphenicol	24(96)	1(4)

Table 3: Multidrug Resistance Pattern Observed Among Non-Typhoidal *Salmonella* (NTS) Isolated from Faecal Samples of Chickens in Ota, Ogun State, Nigeria

Multidrug resistance pattern	No. of resistant Salmonella enterica serovars (%)
CIP-CTX-AMP	1(4)
GEN-AMP	1(4)
GEN-TET-CIP-AMP	1(4)
AMC-CIP-STX-CTX	1(4)
TET-STX-CTX-AMP	1(4)
AMC-CTX-AMP	1(4)
SXT-CTX	1(4)

AMP: Ampicillin, CIP: Ciprofloxacin, GEN: Gentamicin, AMC: Amoxicillin-Clavulanic Acid, CTX: Cefotaxime, CHL: Chloramphenicol, TET: Tetracycline, SXT: Trimethoprim/Sulfamethoxazole

Table 4: Distribution of Assayed Genes in Non-Typhoidal *Salmonella* (NTS) Isolated from Faecal Samples of Chickens in Ota, Ogun State, Nigeria

S/N.	Isolates ID	*invA*	*ttrA*	*fliR*
1.	FI1	POS	POS	NEG
2.	FI2	POS	NEG	NEG
3.	FI3	POS	POS	POS
4.	FI4	POS	NEG	POS
5.	FI5	POS	POS	NEG
6.	FI6	POS	NEG	NEG
7.	FI7	POS	POS	NEG
8.	FI8	POS	POS	POS
9.	FI9	POS	POS	NEG
10.	FI10	POS	NEG	NEG
11.	FI11	POS	NEG	POS
12.	FI12	POS	NEG	NEG
13.	FI13	POS	NEG	POS
14.	FI14	POS	NEG	NEG
15.	FI15	POS	NEG	POS
16.	FI16	POS	POS	POS
17.	FI17	POS	NEG	NEG
18.	FI18	POS	NEG	NEG
19.	FI19	POS	NEG	NEG
20.	FI20	POS	NEG	NEG
21.	FI21	POS	NEG	NEG
22.	FI22	POS	NEG	NEG
23.	FI23	POS	NEG	NEG
24.	FI24	POS	NEG	NEG
25.	FI25	POS	NEG	NEG
26.	*S. typhimurium* 14028	POS	POS	POS
27.	*E. coli*	NEG	NEG	NEG
28.	*P. aeruginosa*	NEG	NEG	NEG
29.	*P. stuartii*	NEG	NEG	NEG
30.	*P. mirabilis*	NEG	NEG	NEG

FI - Faecal Isolates; POS - Positive; NEG - Negative; *S. typhimurium* 14028 - Positive Control Sample; *P. aeruginosa*, *P. stuartii* and *P. mirabilis*- Negative Control Samples

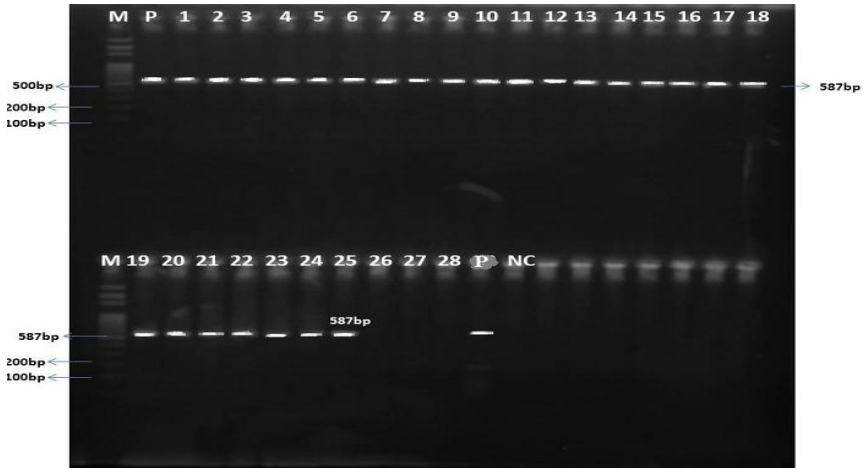

Figure 2: Gel Image Showing Positive 587 bp Bands of *invA* Gene. M: 100 bp DNA Molecular Marker.Lanes 1 - 25 Positive for *invA*; 26 - 28 Negative for *invA*. P: Positive Control; NC: Negative Control

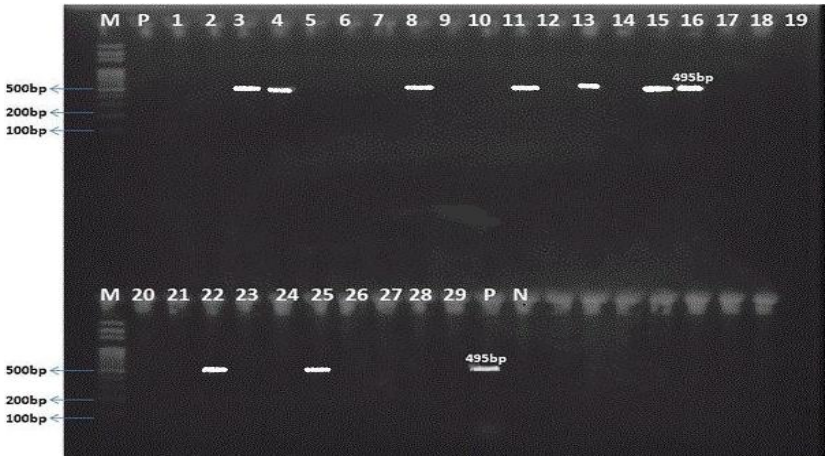

Figure 3: Gel Image showing Positive 495 bp Bands of *filR* gene. M: 100 bp DNA Molecular Marker. Lanes 3, 4, 8, 11, 13, 15, 16, 22 and 25 Positive for *fliR*; 26 - 29 Negative for *fliR*. P: Positive Control; NC: Negative Control

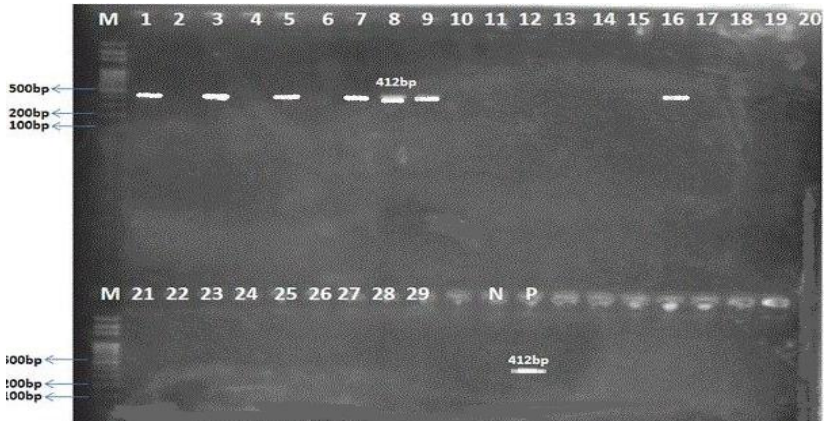

Figure 4: Gel Image showing Positive 412 bp Bands of *ttrA* Gene. M: 100 bp DNA Molecular Marker. Lanes 1, 3, 5, 7, 8, 9 and 16 Positive for *ttrA*; 26 - 29 Negative for *ttrA*. P: Positive Control; NC: Negative Control