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RESEARCH ARTICLE

Investigating the Bacteriological Contamination after Artificial Insemination in Early, Mid and Late Production Period in Broiler Breeder

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A B S T R A C T

The present study was designed to investigate the impact of breeding methods like artificial insemination (AI) vs natural mating (NM) and body weight variations on bacterial contamination and antimicrobial resistance (AMR) in broiler breeders. Ross-308 broiler breeder hens (n=3000) and males (n=255) were selected for this study. Out of those 255 male birds 150 (10%) were selected for natural mating and 105 (7% of hens) for artificial insemination groups. Both male and female groups were further subdivided into three weight categories sub-groups; underweight (UW), standard weight (SW) and overweight (OW). Fifteen (3%) hens out of 500 hens from each weight group were randomly selected and slaughtered to get samples for bacteriological and molecular analysis. A total 18.89% and 35.92% samples were found positive for *Salmonella* and *E. coli* respectively. Results revealed that the bacteria like *Salmonella pullorum-gallinarum* and *E. coli* were found significantly higher in AI groups as compared to NM groups. It was also found that at late life cycle (60th week of age), the presence of *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), *Salmonella pollurum-gallinarum*, and *E. coli* were significantly higher than that of lower age groups. The results also indicated that there is a comparatively lesser chance of *MG*, *MS*, *E. coli,* and *Salmonella pollurum-gallinarum* at the 30th week of age as compared to 45th and 60th weeks of age. On the basis of antibiotic sensitivity testing results; Oflaxacine and colistin were found susceptible among 23 tested antibiotics. In conclusion, it was observed that the chance of bacterial contamination increases with the increase of age in AI groups.

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INTRODUCTION

Artificial insemination is one of the key aspects of any breeding program now a day as the prime objective of this technique is to get the maximum number of hatch- able eggs and day-old chicks through optimal fertility (Shaheen *et al.*, 2021). However, AI is the cause of some bacterial contamination like *E. coli*, *M. gallisepticum* (MG), *M. synoviae* (MS) and *S. palluram-gallinarum* (Seifi and Shirzad, 2012). The main factors responsible for bacterial contamination during AI would be dry litter, contaminated equipment and overall unhygienic conditions (Habibullah *et al.,* 2015; Cyriac and Joseph, 2022). Along with other factors during AI, the vent is exposed to the outside environment which ultimately prompts to carry bacterial infections. In the late period of egg-laying e.g. 60 weeks of age or 45 weeks onwards, there are big chances of contamination particularly *E. coli* and *Salmonella* as compared to early life laying due to decline in immunity. *E. coli* is the most widespread cause of contamination, especially in artificially inseminated poultry birds on floor, which results in severe health problems and economic losses during production performance (Hashem *et al.,* 2020).

Litter was considered as floor or bedding for the breeder house (Aviagen, 2016), however, the use of litter can also cause bacterial contamination in chickens, if not

handled properly. Technical points must be considered including breeding of healthy birds to minimize the chances of bacterial infection, leading to improve performance of the flock (Petek *et al.,* 2014). Another source is pathogenic bacteria present in the male and female digestive tract which might be transmitted through artificial insemination in both parent flock and progeny (Wang *et al.,* 2013; Borges *et al.,* 2014). Natural mating is quite better and safe as compared to AI as suggested by Blanco and Höfle (2004) and Shaheen *et al.*, (2021). However, in NM, the chances of vertical and horizontal transmission of diseases cannot be ruled out as some gastrointestinal microbes might be incorporated at the time of copulation (Kabir, 2010). Careful consideration is needed in poultry birds to minimize the risk of horizontally and vertically transmitted diseases as the fate of the broiler flock depends entirely on the prevalence of these diseases (Shaheen *et al.,* 2020). Another most important aspect is chick's hygiene which is greatly affected by the method of insemination (Li *et al.,* 2016). Additionally, contaminants used in poultry farming pose adverse effects by hampering post-hatch performance and deteriorating the meat quality. Also poor quality meat as a result of using the contaminants poses negative impacts on consumer preference for chicken meat (da Silva *et al.,* 2017). Another aspect of using AI is that it interferes with egg geometry which might lead to poor quality chicks (Sayyazadeh and Shahsavarani, 2005; De Reu *et al.,* 2009). Poor quality chicks are more prone to various diseases and therefore adversely affect the growth of the entire flock; resulting in lower livability and ultimately greater cost of production (Wang *et al.,* 2018). Additionally, bacterial infections like *E. coli*, *Salmonella Pullorum* (SP), and *Mycoplasma gallisepticum* (MG) are considered the utmost cause of mortality and these are also among the most common problems in the broiler industry (Cox *et al.,* 2002; Wang *et al.,* 2018). Omphalitis is caused by *E. coli* and *Salmonella* which is identified as the vital cause of mortality in an early chick's life. Moreover, *E. coli* and *Salmonella* infections spread from chick to chick during brooding and hatchery as these pathogens proliferate in the intestine of newly hatched infected chicks during embryogenesis, Although, AI is of utmost importance as it is an obligatory and most efficient tool in the caged flock and it is also important as it inhibits the decline in fertility of parent flock (floored flock), its retro hygienic impacts are yet to be explored in broiler breeders as compared to NM both in caged and floored parent flock because the quality in term of production efficiency and reduction in cost is of paramount importance in commercial poultry (Saleemi *et al.,* 2018). Therefore, the current study is designed to fulfill this literature gap by understanding the impacts of AI and/or NM on broiler performance when the parent flock is being kept in different production systems (Floor and Cage) during its peak and post-peak production phases.

Infectious diseases remained a continuous threat in poultry production systems since ancient times (Abbas *et al.,* 2017; Mahmood *et al.,* 2018; Saleemi *et al.,* 2018; Zhang *et al.,* 2020). Mycoplasmosis and Salmonellosis are the most important diseases in chickens. Chronic respiratory disease (CRD) is caused by *Mycoplasma gallisepticum* (MG) in chickens (Shah, 2018). Poultry birds of all ages got affected by MG, however, young birds are more susceptible to this pathogen (Shoaib *et al.,* 2019). The transmission of MG occurs both by vertical and horizontal means. Respiratory sounds, coughing, nasal and ocular discharge conjunctivitis, and a drop in egg production are among the most important clinical signs of MG infection (Shah, 2018). *Mycoplasma synoviae* (MS) causes upper respiratory tract infections which ultimately damage the air sacs and also enhance the chances of secondary respiratory infections caused by other pathogens (Seifi and Shirzad, 2012). In addition to respiratory problems, MS also causes joint abnormalities and loss of egg production in chickens which ultimately leads to lower egg production. The transmission of MS occurred both by horizontal and vertical means and this infection persists as a sub-clinical form in the flock (Heleili *et al.,* 2012). Diagnosis of MS infection can be made clearly based on clinical signs, serology, and epidemiological findings followed by isolation of bacteria through culturing techniques (Shoaib *et al.,* 2019). Two important bacterial pathogens i.e. *S. gallinarium* and *S. pullorum* cause Fowl typhoid and Pullorum disease in poultry which is very common and causes huge economic losses in the poultry industry (Ansari *et al.,* 2017). Due to the expansion of the poultry sector both these diseases i.e. fowl typhoid and Pullorum disease has become a common and widespread problem nowadays. Transmission of these diseases occurred by vertical means which means the spread of infections from egg to embryos. Diagnosis of *S. pullorum* and *S. gallinarium* is made on the basis of culturing and serological tests. The serum agglutination test is used to find the prevalence of infection and detection of the infected flocks (Sarkar *et al.,* 2005). Keeping in view the economic importance of *E. coli*, MG, MS, and *Salmonella pollurum-gallinarum*, this study is designed to determine the seroprevalence of all four pathogens in different weight categories of chicken under the influence of natural mating or artificial insemination.

MATERIALS AND METHODS

Ross-308 broiler breeder hens (n=3000) and males (n=255) were selected for this study. Out of those 255 male birds 150 (10% of laying hens) were selected for natural mating (NM) group and 105 for artificial insemination (AI) group which designated a total percentage of 7% of female birds. Hens were also divided into two major treatment groups NM (n=1500) and AI (n=1500) groups. Both male and female groups were further subdivided into three weight categories sub-groups; underweight (UW), standard weight (SW) and overweight (OW) with 500 hens in each subgroup. Each sub-group was consisted of five replicates, each with 100 breeding hens per replicate and the male and female ratios were 1:10 in NM and 1:7 in AI group. Fifteen hens (3%) out of 500 hens from each sub-group were randomly selected and slaughtered to get samples for bacteriological study. For *Salmonella* and *E. coli* tissue samples were collected and for Mycoplasma blood serum samples were collected for the plate agglutination test. Tissue samples were taken from the liver, trachea, lungs, ovaries, and spleen of broiler breeder slaughtered hens in an aseptic environment at KK Chicks breeder farm Chakwal.

Isolation, Identification and Confirmation of *Salmonella* and *E. coli:* All the samples were streaked on MacConkey's agar medium (Oxoid Ltd., Basingstoke, Hampshire, England) plates in the laboratory and incubated at 37°C overnight. *Salmonella* suspected yellow colonies and *E. coli* suspected pink colonies were picked from the MacConkey agar plates and were streaked on xylose lysine deoxycholate (XLD) and EMB agar medium respectively and incubated overnight at 37°C. Bacteriological identifications of *Salmonella* and *E. coli* were completed through Gram staining. Slides were prepared according to the standard method of gram staining technique and observed under a microscope.

Molecular identification of *Salmonella* and *E. coli* isolates

DNA extraction: The purified Salmonella culture grown on XLD and *E. coli* culture on EMB agar were used for DNA isolation. The genomic DNA of the isolates was purified using Wiz Prep TM genomic DNA kit (Seongnam, South Korea).

PCR (Polymerase Chain Reaction): PCR was performed for detection of invA gene (521bp) and *mal*-B gene (580bp) in all Salmonella and *E. coli* confirmed isolates respectively. Species-specific primers (F-5'-TTGTTAC GGCTATTTTGACCA-3' and R-5-CTGACTGCTACCTT GCTGATG-3') were used to amplify the invA gene of Salmonella (Seedy *et al.,* 2016). Species-specific primers (Eco-F-5'-GACCTCGGTTTAGTTCACAGA-3' and Eco-R-5'-CACACGCTGACGCTGACCA-3) were used to amplify the *mal*-B gene of *E. coli.*

Gel electrophoresis: The amplified PCR products were analyzed using 1.8% agarose gel (stained with ethidium bromide) at 90 Voltage for 70 minutes and visualized by Gel Documentation System.

Sequencing analysis of PCR product: One of the positive PCR products of both Salmonella and *E. coli* was sent for sequencing to Macrogen (Korea) for the confirmation of the Inv-A and *mal*-B genes sequences respectively. The gene sequences were subjected to blast search in the GenBank.

Antimicrobial susceptibility testing: All samples positive for Salmonella and *E. coli* recovered from slaughtered birds were tested for their antimicrobial susceptibility to 23 different antimicrobial discs by the Kirby Bauer disc diffusion method, and the results were expressed as susceptible, intermediate, or resistant according to Clinical and Laboratory Standards Institute (CLSI) guidelines (Humphries *et al.*, 2018). The antibiotics used for the antibiotic susceptibility testing were as follows: ciprofloxacin (5 µg), gentamycin (10 µg), ceftrioxone (30 µg), amoxicillin (10 µg), doxycycline (30 µg), enrofloxacin (5 ug), vancomycin (30 µg), amoxy + clauvalanic (30 µg), streptomycin(10 µg), cefixime (5 µg), amikacin (30 µg), colistin sulphate (10 ug), ceftazidime (30 µg), cefoxitin (30 g), tetracycline (30 µg), trimethoprim + sulphamethoxizole (1.25 +23.75 µg), neomycin (30 µg), trimethoprim (5 ug), gentamycin (30 µg), levofloxacin (5 µg), ofloxacin (5 µg), excenel (30 µg), oxytetracycline (30 µg). The Salmonella and *E. coli* were grown in 5 mL MacConkey broth at 37°C for 8 hours. Sterile Mueller Hinton Agar (MHA) plates were used to prepare uniform bacterial lawn using a cotton swab with the help of an aseptic technique. These antibiotic discs were kept on MHA plates by ensuring sufficient spacing between the plates using sterilized forceps. The plates were incubated at 37°C overnight and the very next day, the zone of inhibition was assessed, and its diameter was recorded in millimeters as per CLSI standards.

Isolation and Identification of Mycoplasma species using Plate agglutination test: The plate agglutination test was performed in a clean place with no direct sunlight and dust exposure. Just prior to performing the test the serum and the antigen were maintained at room temperature (25°C) before use and vials were gently shaken. The MG and MS plate agglutination tests were performed separately by using one drop of MG or MS antigen (Ceva Biovac) and a drop of test serum.

Statistical analysis: The data were analyzed by a two-way ANOVA test, with mating and body weight as the main effects. The graphical results were expressed as mean ± standard deviation (M ± SD). Results with $p < 0.05$ were considered statistically significant. When a significant main effect was observed, Tukey's test was used to compare the differences among groups.

RESULTS

Bacteriological Identification of Salmonella, *E. coli* and Mycoplasma from samples: Organisms showing yellow color colonies on MacConkey agar indicating the non-lactose fermenter was chosen and further purified on XLD agar. A total 51 (18.89%) samples found positive for salmonella out of 270 (Table 1). The positive samples showing characteristics of black colonies due to H_2S gas production was chosen for molecular identification.

For the isolation and identification of *E. coli*, samples were taken from sick birds and subjected to bacteriological analysis. A total of 97 out of 270 (35.92%) samples were found positive ((Table 1) and have shown yellow color colonies on MacConkey agar indicating the non-lactose fermenters. The samples (pink colonies) were further purified on EMB agar medium. On the EMB agar, all the samples have shown green metallic-colored colonies.

Plate agglutination tests were performed for the diagnosis of Mycoplasma infection. Results showed 64 (23.70%) samples positive for MG and 62 (22.96%) for MS (Table 1).

Data reveals that the bacteria like Salmonella *(Salmonella pollurum-gallinarum*) and *E. coli* were found significantly ($P < 0.05$) higher in AI groups as compared to NM groups at early life cycle (30th week of age). The effect of MG and MS was found non-significant ($P > 0.05$) in major treatment groups like AI and NM at the age of 30 weeks of age. The effect of minor treatments like OW, SW, UW on bacteria like *Salmonella pollurum-gallinarum* and *E. coli*, MS and MG was found non-significant ($P > 0.05$) in all treatment groups. Similarly, the data at mid-life cycle (45th week of age) found significant ($P < 0.05$) for MG, MS, and *E. coli* in major treatments groups (AI vs NM) and in minor treatment groups (OW, SW, UM). The *Salmonella*

Table 1: Bacteriological descriptive table for all age groups (30, 45 & 60 weeks) in broiler breeder

Age	Variable	AI			NM			*P*-value		
		OW	SW	UW	OW	SW	UW	Maj Trt	Minor Trt	M1×M2[1]
30	Mg	20 ± 8.16	20 ± 8.16	13.3 ± 8.16	13.3 ± 8.16	6.67 ± 6.67	6.67 ± 6.67	0.17	0.691	0.883
Week	Ms	20 ± 8.16	20 ± 8.16	20 ± 8.16	13.3 ± 8.16	6.67 ± 6.67	6.67 ± 6.67	0.09	0.883	0.883
	E. coli	33.3 ± 0	26.7 ± 6.67	26.7 ± 6.67	20 ± 8.16	13.3 ± 8.16	13.3 ± 8.16	0.027	0.549	1
	Salmonella pollurum-gallinarum	20 ± 8.16	13.3 ± 8.16	20 ± 8.16	6.67 ± 6.67	0 ± 0	6.67 ± 6.67	0.027	0.549	1
45	Mg	46.7 ± 8.16^{a}	26.7± 6.67^{ab}	26.7 ± 6.67^{ab}	20 ± 8.16^{b}	6.67 ± 6.67^{b}	6.67 ± 6.67^{b}	0.001	0.044	0.868
Week	Ms	40 ± 12.5^{a}	20 ± 8.16^{ab}	26.7 ± 6.67^{ab}	13.3 ± 8.16^{ab}	20 ± 8.16^{ab}	6.67 ± 6.67^{b}	0.037	0.049	0.291
	E. coli	53.3 ± 8.16^{a}	46.7 ± 8.16^{ab}	46.7 ± 8.16^{ab}	26.7 ± 6.67^{bc}	13.3 ± 8.16^{c}	20 ± 8.16^{c}	<0.001	0.047	0.89
	Salmonella pollurum-gallinarum	20 ± 8.16	13.3 ± 8.16	26.7 ± 6.67	13.3 ± 8.16	6.67 ± 6.67	6.67 ± 6.67	0.08	0.593	0.593
60	Mg	66.7 ± 10.5^{a}	33.3 ± 10.5^{bc}	46.7 ± 8.16^{ab}	33.3 ± 10.5^{bc}	13.3 ± 8.16^{c}	20 ± 8.16^{bc}	0.002	0.03	0.781
Week	Ms	60 ± 12.5^{a}	33.3 ± 10.5^{ab}	40 ± 12.5^{ab}	26.7 ± 12.5^{ab}	26.7 ± 12.5^{ab}	13.3 ± 8.16^{b}	0.027	0.038	0.496
	E. coli	86.7 ± 8.16^{a}	53.3 ± 13.3^{b}	86.7 ± 8.16^{a}	40 ± 6.67^{bc}	20 ± 8.16^{c}	20 ± 8.16^{c}	<0.001	0.023	0.199
	Salmonella pollurum-gallinarum	53.3 ± 13.3^{ab}	20 ± 8.16^{c}	60 ± 12.5^{a}	26.7 ± 12.5^{bc}	13.3 ± 8.16^{c}	13.3 ± 8.16^{c}	0.006	0.048	0.197

Values are means ± SEM, n = 15 per treatment group (values are 3% of total number of birds in positive percentile).: $^{a-a}$Means in a row without a common superscript letter differ (P<0.05) as analyzed by two-way ANOVA and the DUNCAN test: [1]M1 × M2 = Maj Trt × Minor Trt interaction effect.

Fig. 1: Disk diffusion test to determine antimicrobial susceptibility testing of isolated *E. coli.*

pollurum-gallinarum value was found non-significant in both major and minor treatments groups. Likewise, the data at late life cycle (60th week of age) found significant (P<0.05) for MG, MS, Salmonella, and *E-coli* in major treatments groups (AI vs NM) and in minor treatment groups (OW, SW, UM).

Molecular identification *Salmonella and E. coli* using PCR: PCR results revealed that the tested isolates were confirmed as being Salmonella Positive samples showed a 520 base pair product of Salmonella invA gene as shown in (Fig. 4).

DNA was extracted from the samples showing green colonies on the EMB agar plate. PCR was performed on the positive samples using *mal*-B gene primers. A band of approximately 580 bp was considered positive. All the samples showed right sized band for the fragment of the *mal*-B gene (Fig. 5).

Sequencing analysis of PCR products: One of the positive products of 520 bp of Salmonella invA gene fragment was sent for DNA sequencing to confirm the PCR results. The DNA sequence obtained has confirmed that the sample belongs to the Salmonella InvA gene (Fig. 4). DNA sequences were further subjected to NCBI BLAST to compare the results with sequences present in the database. A 98% similarity was found with the sequences available in the NCBI database including MW567498 (98%), MW881231 (98%), CP032493 (98%) and CP063710 (98%). The results have shown that the isolated Salmonella samples have a close resemblance with the isolates present worldwide (Fig. 6).

One of the positive products of 580 bp of *E. coli mal*-B gene was also sent for sequencing to confirm the PCR results. The DNA sequence obtained has confirmed the sequence of the *E. coli mal*-B gene fragment. DNA sequences were further subjected to NCBI blast to compare the results with sequences present in the database (Fig. 5). A similarity of up to 99% was found with the sequences available in the NCBI database including CP060057 (99%), CP060061 (99%), CP041422 (98%) and CP047571 (98%) (Fig. 7).

Antibiotic susceptibility testing: Results of antibiotic sensitivity tests revealed that 2 out of 23 antimicrobial agents were found highly sensitive against Salmonella isolates. Those antimicrobials are Cefotaxime, Cefixime, Amakacin, Colistine, ceftriaxone, and Ofloxacin (Fig. 2). On the other hand, high resistances were observed against Gentamycin, Amoxycycline, Doxycycline, Neomycin, Streptomycin, Tetracycline, Vancomycin, Amoxy+Clavulanic acid, Cefoxitin, trimethoprim, and oxytetracycline. ceftiofur, ceftazidime, Amoxycillin, vancomycin, amoxy+clauvalanic, cefoxitin, and colistin. On the basis of the results findings, Oflaxacine and colistin were selected for the treatment.

Results of in-vitro sensitivity tests of Salmonella against 23 antimicrobial agents revealed that *E. coli* showed high sensitivities (based on zone of inhibitions as per CSLI guidelines) (Humpries *et al.*, 2018) against Ciprofloxaacine, Cefixime, Amakacin, Colistine, ceftriaxone, Ofloxacin and Levofloxacin (Fig. 1-3). On the other hand, high resistances were observed against Gentamycin, Amoxycycline, Neomycin, Streptomycin, Tetracycline, Vancomycin, Amoxy+Clavulanic acid, Cefoxitin, trimethoprim, and oxytetracycline. ceftiofur, ceftazidime, Amoxycillin, vancomycin, amoxy+clauvalanic, cefoxitin. Intermediate sensitivity was observed against Doxycycline, Ceftaxidine, and Ceftiofur. Thus, Oflaxacine and colistin were used for the treatment as represented in Fig. 1-3.

DISCUSSION

The results of present trials indicated that there is less chance of MG, MS, *E. coli* and Salmonella infections at the 30th week of age as compared to the 45th and 60th week of age which is due to the reason that the chances of bacterial contamination increase with the increase of age.

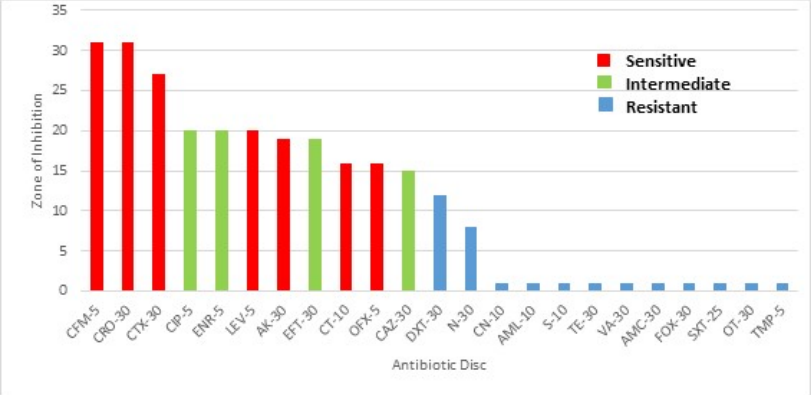


Fig. 2: Graph showing antibiotic sensitivity testing against Salmonella on the basis of measurements of zones of inhibitions (mm).

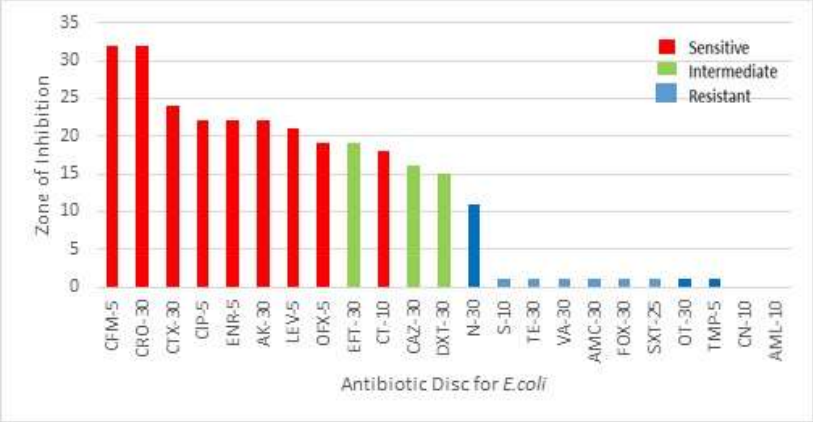


Fig. 3: Graph showing antibiotic sensitivity testing against *E. coli* on the basis of measurements of zones of inhibitions (mm).

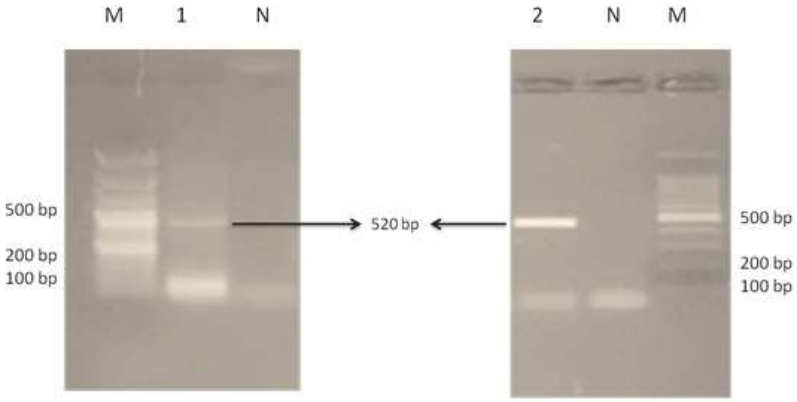


Fig. 4: PCR amplification of *Salmonella* InvA gene. Positive results showing a band of approximately 520 base pair. Lane P: Positive control, Lanes 1 and 2: test samples, Lane M: 100bp DNA marker, N: Negative control

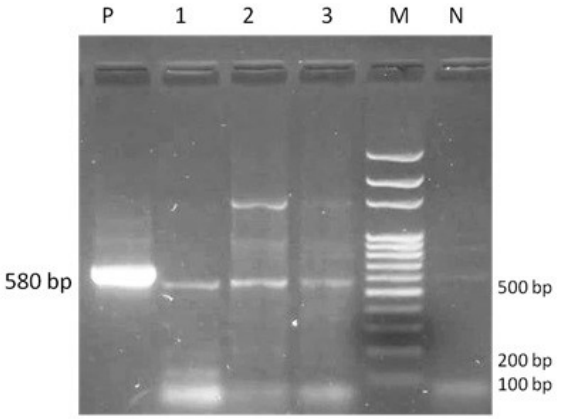
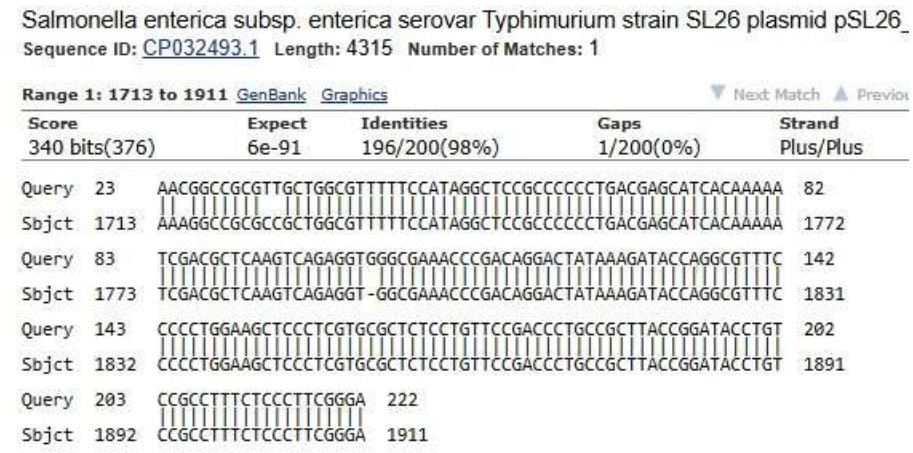


Fig. 5: PCR amplification of *E. coli mal*-B gene fragment. Positive results showing a band of approximately 580 base pair. Lane P: Positive control, Lanes 1-3: test samples, Lane M: 100bp DNA marker, N: Negative control.

The findings of present trials regarding the AI are supported by (Corrier *et al.*, 1999; Dhama *et al.,* 2014; Ahmed *et al.*, 2015) who reported that AI plays a key role in the transmission of bacterial diseases but the same finding is contradicted with the results at the age of 30th week. So, in a conclusion, age has a major effect on disease transmission.

Despite the availability of the modern-day molecular identification techniques, biochemical test is still use for the preliminary identification of microorganism. Strain to strain difference can be seen in morphological test and other biochemical assays but these tests are not of much help in identifying species specific strains. For isolated strains molecular study can be used to find similarity with already identified specific species whose data are available in online libraries. As compare to previous techniques such as morphological and biochemical test, molecular identification is now considered more as more effective.



Salmonella enterica subsp. enterica serovar Typhimurium strain SL26 plasmid pSL26_
Sequence ID: CP032493.1 Length: 4315 Number of Matches: 1

Range 1: 1713 to 1911 GenBank Graphics Next Match Previou

Score	Expect	Identities	Gaps	Strand
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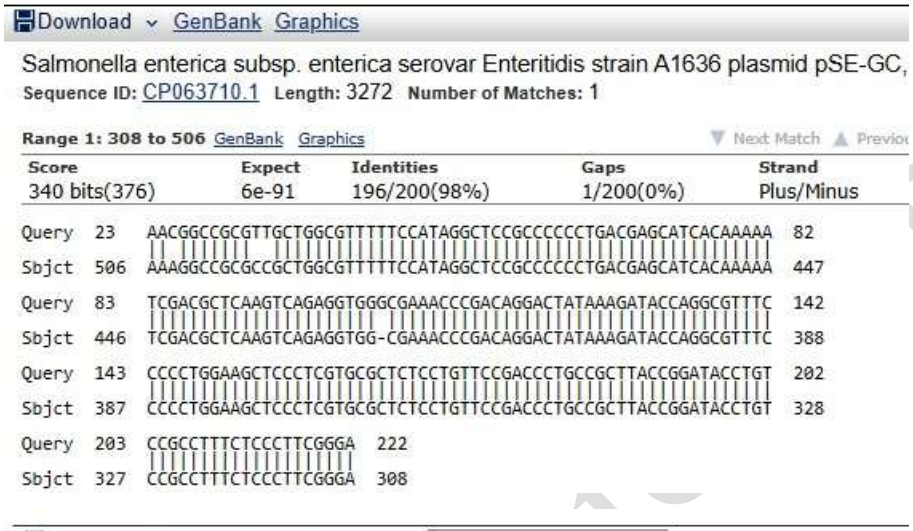
Fig. 6: Similarity in sequence of the salmonella from the present study with accession no. MW567498 (98%) and MW881231 (98%) using NCBI Blast.



Download GenBank Graphics

Salmonella enterica subsp. enterica serovar Enteritidis strain A1636 plasmid pSE-GC,
Sequence ID: CP063710.1 Length: 3272 Number of Matches: 1

Range 1: 308 to 506 GenBank Graphics Next Match Previou

Score	Expect	Identities	Gaps	Strand
340 bits(376)	6e-91	196/200(98%)	1/200(0%)	Plus/Minus

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             || |||||||  ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  506   AAAGGCCGCGCCGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAA  447

Query  83    TCGACGCTCAAGTCAGAGGTGGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTC  142
             ||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  446   TCGACGCTCAAGTCAGAGGTGG-CGAAACCCGACAGGACTATAAAGATACCAGGCGTTTC  388

Query  143   CCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGT  202
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  387   CCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGT  328

Query  203   CCGCCTTTCTCCCTTCGGGA  222
             ||||||||||||||||||||
Sbjct  327   CCGCCTTTCTCCCTTCGGGA  308
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Download GenBank Graphics Sort by: E value

Escherichia coli strain G8 chromosome, complete genome
Sequence ID: CP060057.1 Length: 5295653 Number of Matches: 7

Range 1: 4897135 to 4897651 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1009 bits(509)	0.0	516/517(99%)	1/517(0%)	Plus/Minus

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Query  33       AAAAGCCAGGGGGTGGAGGATTTAAGCCATCTCCTGATGACGCA-AGTCAGCCCATCATG  91
                ||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Sbjct  4897651  AAAAGCCAGGGGGTGGAGGATTTAAGCCATCTCCTGATGACGCATAGTCAGCCCATCATG  4897592

Query  92       AATGTTGCTGTCGATGACAGGTTGTTACAAAGGGAGAAGGGCATGGCGAGCGTACAGCTG  151
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4897591  AATGTTGCTGTCGATGACAGGTTGTTACAAAGGGAGAAGGGCATGGCGAGCGTACAGCTG  4897532

Query  152      CAAAATGTAACGAAAGCCTGGGGCGAGGTCGTGGTATCGAAAGATATCAATCTCGATATC  211
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4897531  CAAAATGTAACGAAAGCCTGGGGCGAGGTCGTGGTATCGAAAGATATCAATCTCGATATC  4897472

Query  212      CATGAAGGTGAATTCGTGGTGTTTGTCGGACCGTCTGGCTGCGGTAAATCGACTTTACTG  271
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4897471  CATGAAGGTGAATTCGTGGTGTTTGTCGGACCGTCTGGCTGCGGTAAATCGACTTTACTG  4897412

Query  272      CGCATGATTGCCGGGCTTGAGACGATCACCAGCGGCGACCTGTTCATCGGTGAGAAACGG  331
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4897411  CGCATGATTGCCGGGCTTGAGACGATCACCAGCGGCGACCTGTTCATCGGTGAGAAACGG  4897352

Query  332      ATGAATGACACTCCGCCAGCAGAACGCGGCGTTGGTATGGTGTTTCAGTCTTACGCGCTC  391
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4897351  ATGAATGACACTCCGCCAGCAGAACGCGGCGTTGGTATGGTGTTTCAGTCTTACGCGCTC  4897292

Query  392      TATCCCCACCTGTCAGTAGCAGAAAACATGTCATTTGGCCTGAAACTGGCTGGCGCAAAA  451
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4897291  TATCCCCACCTGTCAGTAGCAGAAAACATGTCATTTGGCCTGAAACTGGCTGGCGCAAAA  4897232

Query  452      AAAGAGGTGATTAACCAACGCGTTAACCAGGTGGCGGAAGTGCTACAACTGGCGCATTTG  511
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4897231  AAAGAGGTGATTAACCAACGCGTTAACCAGGTGGCGGAAGTGCTACAACTGGCGCATTTG  4897172

Query  512      CTGGATCGCAAACCGAAAGCGCTCTCCGGTGGTCAGC  548
                ||||||||||||||||||||||||||||||||||||
Sbjct  4897171  CTGGATCGCAAACCGAAAGCGCTCTCCGGTGGTCAGC  4897135
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Fig. 7: Similarity in sequence of the *E. coli* isolated in the present study with accession no. CP060057 (99%) using NCBI Blast.

Through many methods, bacterial strains can be identified but now-a-days the criteria of correct and precise bacterial identification are based on analysis of gene sequencing (Kim *et al.*, 2012) which also provide information about pathogenic potential of bacteria. In a study done by Ahmed *et al.*, (2015) 30 semen samples tested were found positive for one or more than one type of bacteria. Out of thirty positive samples *E. coli* was isolated from 18 samples and *Salmonella enteritidis* was isolated form 9 samples. In the present study invA gene and *mal*-B genes were also used to identify pathogenic Salmonella and *E. coli* respectively. The DNA sequence similarities revealed that isolated strains were 98-100% similar to the sequences of Salmonella enteric sub species enteric and pathogenic *E. coli* present worldwide (Donoghue *et al.,* 2004; Dhama *et al.*, 2014).

From last decade the race of getting a greater number of chicks per hen housed is increasing day by day, from heavy breeds it is very difficult to maintain hatchability specially during late laying phases of broiler breeder, so assisted reproductive technology technique in the form of AI has been getting more popularity. But due to poor hygiene there are challenge like bacterial contamination, which not only effects parent stock, but also deteriorates the performance of progeny as well. No doubt, by adopting AI technique poultry farmers are getting 10-15 more number of chicks per hen as shown in our own trial. So, we recommend especial attention towards disinfection, hygiene, and health monitoring of the flocks during AI.

Disease transmission at a later age is an indicator of the low immunity level of birds. Another factor may be the wet litter. In general, during mating, the whole reproductive and digestive tract is incorporated with insemination fluid, and it might be one of the reasons for contamination even in breeding hens (Blanco and Höfle, 2004; Kabir, 2010). So, there are many other factors that contribute to bacterial contamination (Li *et al.,* 2018). The advantages of AI are many which support the wide adaptation of this technique in the poultry industry to its betterment. Making AI as an integral part of chickens' life would facilitate the process by saving time and economy however there is a risk of possible transmission of various pathogenic organisms through semen or during the process of insemination. It is necessary to use sterile technique during AI and regular screening like antibiotic sensitivity testing or molecular diagnosis using PCR will help to check the spread of several diseases.

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Author's Contribution: Nasir Mukhtar and Aayesha Riaz designed and directed the project; Farhan Farooq performed the experiments and wrote the manuscript with support from Nasir Mukhtar and Aayesha Riaz. All authors discussed the results and contributed to the final manuscript. Zaib Ur Rehman helped in trail design and data analysis and revised the manuscript, and Tanveer Ahmad guided how to manage the trails and replicates, while Arfan Yousaf and Javid Iqbal guided the overall farm management.

Conflict of Interest: The authors declare no conflict of interest.

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