



RESEARCH ARTICLE

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

Farhan Farooq¹, Aayesha Riaz², Zaib Ur Rehman¹, Arfan Yousaf³, Javid Iqbal⁴, Tanveer Ahmad⁵ and Nasir Mukhtar^{5*}

¹Department of Poultry Sciences, PMAS Arid Agriculture University Rawalpindi Pakistan

²Department of Parasitology & Microbiology, PMAS Arid Agriculture University Rawalpindi Pakistan

³Department of Clinical Studies, PMAS Arid Agriculture University Rawalpindi Pakistan

⁴University of Agriculture, Faisalabad, Pakistan (TTS Campus)

⁵Department of Livestock Production and Management, PMAS Arid Agriculture University Rawalpindi Pakistan

*Corresponding author: nmukhtar@uair.edu.pk; dr_farhanf@hotmail.com

ARTICLE HISTORY (22-154)

Received: April 24, 2022
Revised: September 6, 2022
Accepted: September 13, 2022
Published online: September 28, 2022

Key words:

Artificial insemination
Natural mating
Breeding hens
Bacterial load
Cultural sensitivity

ABSTRACT

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 pullorum-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 pullorum-gallinarum* at the 30th week of age as compared to 45th and 60th weeks of age. On the basis of antibiotic sensitivity testing results; Oflaxacin 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.

To Cite This Article: Farooq F, Riaz A, Rehman ZU, Yousaf A, Iqbal J, Ahmad T and Mukhtar N, 2023. Investigating the bacteriological contamination after artificial insemination in early, mid and late production period in broiler breeder. Pak Vet J, 43(1): 17-24. <http://dx.doi.org/10.29261/pakvetj/2022.068>

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. pullorum-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 pullorum-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™ 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'-TTGTTACGGCTATTTTGACCA-3' and R-5'-CTGACTGCTACCTTGCTGATG-3') were used to amplify the *invA* gene of *Salmonella* (Seedy *et al.*, 2016). Species-specific primers (Eco-F-5'-GACCTCGTTTGTAGTTCACAGA-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), ceftriaxone (30 µg), amoxicillin (10 µg), doxycycline (30 µg), enrofloxacin (5 µg), vancomycin (30 µg), amoxy + clauvalanic (30 µg), streptomycin (10 µg), cefixime (5 µg), amikacin (30 µg), colistin sulphate (10 µg), ceftazidime (30 µg), ceftoxitin (30 µg), tetracycline (30 µg), trimethoprim + sulphamethoxazole (1.25 +23.75 µg), neomycin (30 µg), trimethoprim (5 µg), 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 H₂S 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 Week	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
	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
	<i>E. coli</i>	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
	<i>Salmonella pollurum-gallinarum</i>	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 Week	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
	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
	<i>E. coli</i>	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
	<i>Salmonella pollurum-gallinarum</i>	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 Week	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 ^c	0.002	0.03	0.781
	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
	<i>E. coli</i>	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
	<i>Salmonella pollurum-gallinarum</i>	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-b}Means in a row without a common superscript letter differ (P<0.05) as analyzed by two-way ANOVA and the DUNCAN test: ¹M1 × 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, Ofloxacin 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 Ciprofloxacin, 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, Ofloxacin 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.

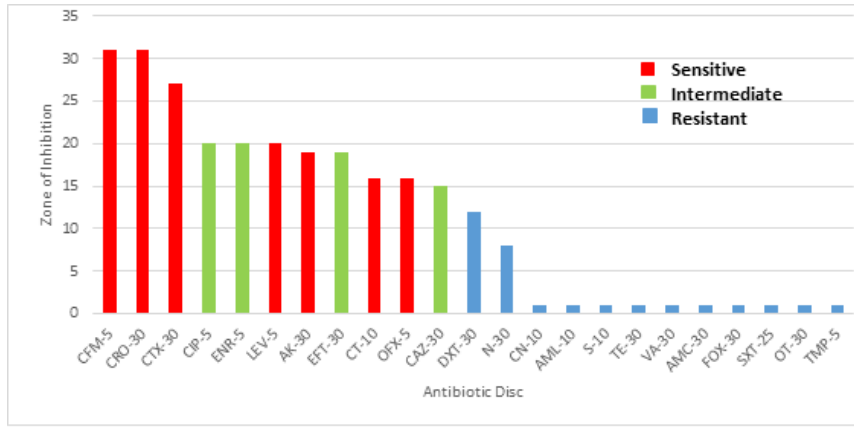


Fig. 2: Graph showing antibiotic sensitivity testing against Salmonella on the basis of measurements of zones of inhibitions (mm).

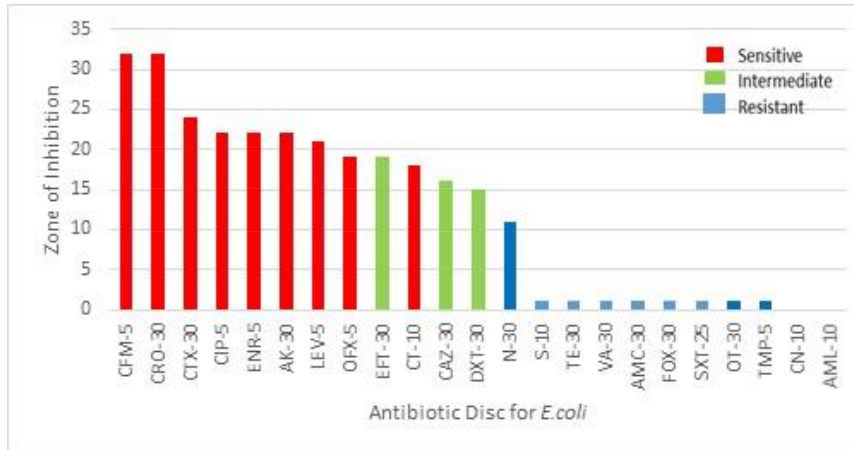


Fig. 3: Graph showing antibiotic sensitivity testing against E. coli on the basis of measurements of zones of inhibitions (mm).

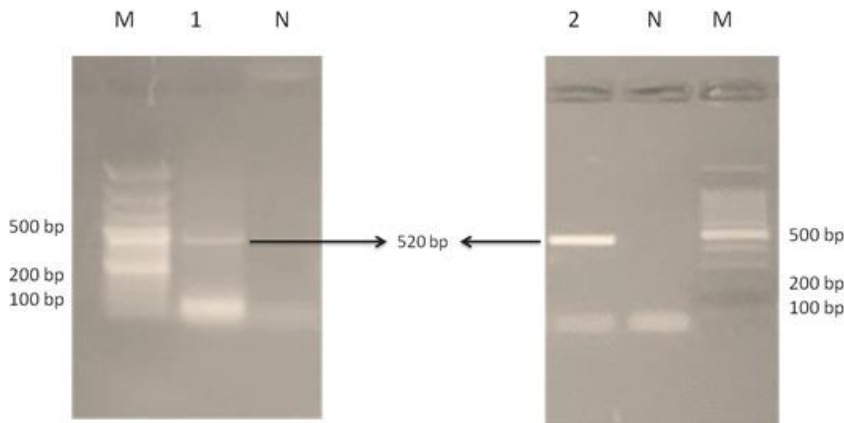


Fig. 4: PCR amplification of Salmonella InvA gene. Positive results showing a band of approximately 520 base pair. Lane P: Positive control, Lanes 1 and 2: test samples, Lane M: 100bp DNA marker, N: Negative control

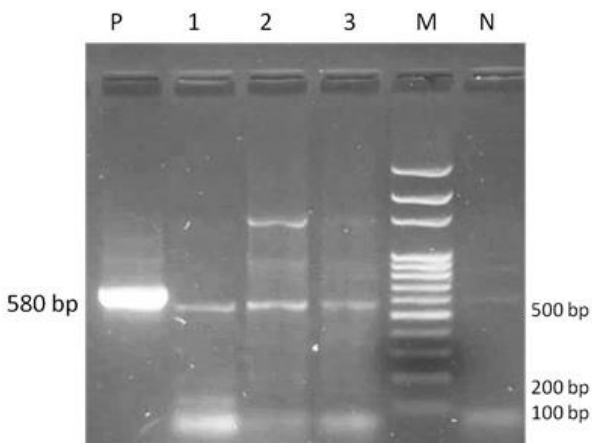


Fig. 5: PCR amplification of E. coli mal-B gene fragment. Positive results showing a band of approximately 580 base pair. Lane P: Positive control, Lanes 1-3: test samples, Lane M: 100bp DNA marker, N: Negative control.

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 ▲ Previous

Score	Expect	Identities	Gaps	Strand
340 bits(376)	6e-91	196/200(98%)	1/200(0%)	Plus/Plus
Query 23	AACGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAA			82
Sbjct 1713	AAAGGCCGCGCCGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAA			1772
Query 83	TCGACGCTCAAGTCAGAGGTGGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTC			142
Sbjct 1773	TCGACGCTCAAGTCAGAGGT-GGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTC			1831
Query 143	CCCCGGAAGCTCCCTCGTGCCTCTCCTGTTCCGACCTGCCGCTTACCGGATACCTGT			202
Sbjct 1832	CCCCGGAAGCTCCCTCGTGCCTCTCCTGTTCCGACCTGCCGCTTACCGGATACCTGT			1891
Query 203	CCGCCTTTCTCCCTTCGGGA	222		
Sbjct 1892	CCGCCTTTCTCCCTTCGGGA	1911		

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 ▲ Previous

Score	Expect	Identities	Gaps	Strand
340 bits(376)	6e-91	196/200(98%)	1/200(0%)	Plus/Minus
Query 23	AACGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAA			82
Sbjct 506	AAAGGCCGCGCCGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAA			447
Query 83	TCGACGCTCAAGTCAGAGGTGGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTC			142
Sbjct 446	TCGACGCTCAAGTCAGAGGTGG-CGAAACCCGACAGGACTATAAAGATACCAGGCGTTTC			388
Query 143	CCCCGGAAGCTCCCTCGTGCCTCTCCTGTTCCGACCTGCCGCTTACCGGATACCTGT			202
Sbjct 387	CCCCGGAAGCTCCCTCGTGCCTCTCCTGTTCCGACCTGCCGCTTACCGGATACCTGT			328
Query 203	CCGCCTTTCTCCCTTCGGGA	222		
Sbjct 327	CCGCCTTTCTCCCTTCGGGA	308		

[Download](#) ▼ [GenBank](#) [Graphics](#) Sort by:

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
Query 33	AAAAGCCAGGGGTGGAGGATTTAAGCCATCTCCTGATGACGCA-AGTCAGCCCATCATG			91
Sbjct 4897651	AAAAGCCAGGGGTGGAGGATTTAAGCCATCTCCTGATGACGCAAGTCAGCCCATCATG			4897592
Query 92	AATGTTGCTGTCGATGACAGGTGTTACAAAAGGAGAAAGGCGATGGCAGCGTACAGCTG			151
Sbjct 4897591	AATGTTGCTGTCGATGACAGGTGTTACAAAAGGAGAAAGGCGATGGCAGCGTACAGCTG			4897532
Query 152	CAAAAATGAACGAAAGCCTGGGGCGAGGTCGTGGTATCGAAAGATATCAATCTCGATATC			211
Sbjct 4897531	CAAAAATGAACGAAAGCCTGGGGCGAGGTCGTGGTATCGAAAGATATCAATCTCGATATC			4897472
Query 212	CATGAAGGTGAATTCGTGGTGTGTCGGACCGTCTGGCTGCGGTAAATCGACTTTACTG			271
Sbjct 4897471	CATGAAGGTGAATTCGTGGTGTGTCGGACCGTCTGGCTGCGGTAAATCGACTTTACTG			4897412
Query 272	CGCATGATTGCCGGGCTTGAGACGATCACCAGCGGCGACCTGTTTCATCGGTGAGAAACGG			331
Sbjct 4897411	CGCATGATTGCCGGGCTTGAGACGATCACCAGCGGCGACCTGTTTCATCGGTGAGAAACGG			4897352
Query 332	ATGAATGACACTCCGCCAGCAGAACGCGGCGTTGGTATGGTGTTCAGTCTTACGCGCTC			391
Sbjct 4897351	ATGAATGACACTCCGCCAGCAGAACGCGGCGTTGGTATGGTGTTCAGTCTTACGCGCTC			4897292
Query 392	TATCCCCACCTGTCAAGTACGAGAAAACATGTCAATTTGGCCTGAAACTGGCTGGCGCAAAA			451
Sbjct 4897291	TATCCCCACCTGTCAAGTACGAGAAAACATGTCAATTTGGCCTGAAACTGGCTGGCGCAAAA			4897232
Query 452	AAAGAGGTGATTAACCAACGCGTTAACCAGGTGGCGGAAGTGCTACAACCTGGCGCATTTG			511
Sbjct 4897231	AAAGAGGTGATTAACCAACGCGTTAACCAGGTGGCGGAAGTGCTACAACCTGGCGCATTTG			4897172
Query 512	CTGGATCGCAAAACCGAAAGCGCTCTCCGGTGGTCAGC	548		
Sbjct 4897171	CTGGATCGCAAAACCGAAAGCGCTCTCCGGTGGTCAGC	4897135		

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 from 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.

Acknowledgement: The authors acknowledge the KK Chicks, Rawalpindi, Pakistan for their support to conduct the trial at their Hatchery and Breeder farm and also acknowledge Dr. Farhan Afzal, Poultry Research Institute Rawalpindi Pakistan to guide and support the trial.

Authors 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.

REFERENCES

- Abbas A, Iqbal Z, Abbas, Saleemi, MK, *et al.*, 2017. *In vivo* anticoccidial effects of *Beta vulgaris* (sugar beet) in broiler chickens. *Microb. Pathog* 111:139-44.
- Ahmed K, Das K and George S, 2015. Bacterial flora of poultry semen and their antibiotic sensitivity pattern. *Int J Appl Pure Sci Agric* 7:39-41.
- Ansari F, Pourjafar H, Bokaie S, Peighambari SM, *et al.*, 2017. Association between poultry density and *Salmonella* infection in commercial laying flocks in Iran using a kernel density. *Pak Vet J* 37:299-304.
- Aviagen, 2016. Aviagen best practice in the breeder house. Accessed October, 2019.
- Blanco J and Höfle U, 2004. Bacterial and fungal contaminants in raptor ejaculates and their survival to sperm cryopreservation protocols. Paper presented at the Proceedings 6th conference of the European Wildlife Disease Association.
- Borges S, Silva J and Teixeira P, 2014. The role of lactobacilli and probiotics in maintaining vaginal health. *Arch Gynecol Obstet* 289:479-89.
- Corrier D, Byrd J, Hargis B, Hume M, *et al.*, 1999. Presence of *Salmonella* in the crop and ceca of broiler chickens before and after preslaughter feed withdrawal. *Poult Sci* 78:45-9.
- Cox N, Stern N and Wilson J, *et al.*, 2002. Isolation of *Campylobacter* spp. from semen samples of commercial broiler breeder roosters. *Avian Dis* 46:717-20.
- Cyriac S and Joseph L, 2022. Incubation and Hatching of Duck Eggs. In *Duck Production and Management Strategies* Springer, Singapore.339-383.
- Da-Silva DCF, de Arruda AMV and Gonçalves AA, 2017. Quality characteristics of broiler chicken meat from free-range and industrial poultry system for the consumers. *J Food Sci Technol J F* 54:1818-26.
- De-Reu K, Rodenburg T and Grijspeerdt K, 2009. Bacteriological contamination, dirt, and cracks of eggshells in furnished cages and noncage systems for laying hens: An international on-farm comparison. *Poult Sci* 88:2442-8.
- Dhama K, Singh R and Mohan J, 2014. Artificial insemination in poultry and possible transmission of infectious pathogens. *Asian J Anim Vet Adv* 9:211-28.
- Donoghue AM, Blore PJ, Cole K, *et al.*, 2004. Detection of *campylobacter* or *salmonella* in turkey semen and the ability of poultry semen extenders to reduce their concentrations. *Poultry Science*, 83:1728-33.
- Habibullah M, Hashem M and Rana M, 2015. Effect of Artificial Insemination on different production parameter in Hubbard classic broiler parent stock. *J Bangladesh Agric Univ* 13:71-7.
- Hashem MA, Neamat-Allah AN, Hammza HE and Abou-Elnaga HM, 2020. Impact of dietary supplementation with *Echinacea purpurea* on growth performance, immunological, biochemical, and pathological findings in broiler chickens infected by pathogenic *E. coli*. *Trop Anim Health Prod* 52:1599-607.
- Heleili N, Ayachi A, Mamache B, *et al.*, 2012. Seroprevalence of *Mycoplasma synoviae* and *Mycoplasma gallisepticum* at Batna commercial poultry farms in Algeria. *Vet World* 5:12.
- Humphries RM, Ambler J, Mitchell SL, *et al.*, 2018. CLSI methods development and standardization working group of the subcommittee on antimicrobial susceptibility testing. CLSI methods development and standardization working group best practices for evaluation of antimicrobial susceptibility tests. *J Clin Microbiol* 56:e01934-17. doi: 10.1128/JCM.01934-17
- Kabir S, 2010. Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *Int J Environ Res Public Health* 7: 89-114.
- Kim OS, Cho YJ, Lee K, *et al* 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* 62: 716-721.
- Li XK, Wang JZ, Wei Xi, *et al.*, 2016. Effect of dietary phosphorus levels on meat quality and lipid metabolism in broiler chickens. *Food Chem* 205:289-96.
- Li Y, Zhan K, Li J, *et al.*, 2018. Comparison of natural mating and artificial insemination on laying performance, egg quality and welfare of fast feathering huainan partridge chickens. *Pak J Zool* 50:1131-5.

- Mahmood MS, Amir HW, Rafique A, 2018. Evaluation of antiviral activity of *Azadirachta indica* (Neem) bark extract against Newcastle disease virus. *Pak Vet J* 38:25-8.
- Petek M, Üstüner H and Yeşilbağ D, 2014. Effects of stocking density and litter type on litter quality and growth performance of broiler chicken. *Kafkas Univ Vet Fak Derg* 20:743-8.
- Saleemi M, Khan M, Ahrar K, et al., 2018. Molecular epidemiology and pathology of chicken infectious anemia in day old broiler chicks in Faisalabad, Pakistan. *Int J Agric Biol* 20:57-61.
- Sarkar S, Rahman M, Rahman M, et al., 2005. Sero-prevalence of *Mycoplasma gallisepticum* infection of chickens in model breeder poultry farms of Bangladesh. *Int J Poult Sci* 4:32-35.
- Sayyazadeh H and Shamsavarani H, 2005. Effects of Artificial Insemination on performance of broiler breeders. *Department of Animal Science, Faculty of Agriculture, Mazandaran University, Sari, Iran. 4th European Poultry Genetics Symposium, Croatia, 2005.*
- Seifi S and Shirzad M, 2012. Seroprevalence and risk factors of *Mycoplasma gallisepticum* infection in Iranian broiler breeder farms. *J Anim Vet Adv* 4:45-8.
- Shah AH, 2018. Seroprevalance of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in commercial broilers and backyard poultry in five districts of Khyber Pakhtunkhwa-Pakistan. *Pak Vet J* 38:2.
- Shaheen MS, Mehmood S and Riaz A, et al., 2020. Effects of different mating strategies in broiler breeder during peak and postpeak phase on subsequent broiler performance. *Poult Sci* 99: 3501-10.
- Shaheen MS, Mehmood S, Mahmud A, et al., 2021. Interventions in mating strategies of broiler breeder during peak and post peak phase influence hatching traits. *Poultry Science*, 100:101095.
- Shoaib M, Riaz A, Malik AM, et al., 2019. Sero-prevalence and associated risk factors of *Mycoplasma Gallisepticum*, *Mycoplasma Synoviae* and *Salmonella Pullorum/Gallinarium* in poultry. *Pak Vet J* 10:29261.
- Wang C, Pors SE and Bojesen AM, 2018. Transmission and pathogenicity of *Gallibacterium anatis* and *Escherichia coli* in embryonated eggs. *Vet Microbiol* 217:76-81.
- Wang Y, Ametaj BN and Gänzle MG, 2013. Characterisation of the bacterial microbiota of the vagina of dairy cows and isolation of pediocin-producing *Pediococcus acidilactici*. *BMC Microbiology*, 13:1-11.
- Zhang K, Li X, Na C, et al., 2020. Anticoccidial effects of *Camellia sinensis* (green tea) extract and its effect on Blood and Serum chemistry of broiler chickens. *Pak Vet J* 40:77-8.