EVALUATION OF MICROBIAL QUALITY AND PATHOGENIC POTENTIALITY OF ENTEROBACTERIA IN POULTRY FEEDS

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Abstract

The present investigation has been carried out to assess the microbial safety and pathogenic potentialities of enterobacteria in poultry feeds. From the results it was observed that total aerobic plate count of poultry feed samples were recorded as $2.8 \times 10^5$ to $5.8 \times 10^9$ cfu/g and 100% samples contained $\geq 10^6$ cfu/g while the highest mean of cfu was counted as log$_{10}$ 8.797/gm. Large number of coliforms were recorded in different poultry feed samples and the ranges of cfu were counted as $1.2 \times 10^5$ to $5.2 \times 10^7$/g while average 75% samples were contaminated with coliform bacteria with $\geq 10^4$ cfu/g and the highest mean of cfu was counted as log$_{10}$ 6.103/g. The ranges of cfu of Escherichia coli were $1.03 \times 10^2$ to $1.09 \times 10^5$/g and 70% samples contained $\geq 10^2$ cfu/g while the highest mean of cfu was counted as log$_{10}$ 4.493/gm. But the ranges of cfu of total Salmonella sp. were recorded as $1.02 \times 10^1$ to $5.25 \times 10^4$ and 50% samples contained $\geq 10^1$ cfu/g and the highest mean of cfu was counted as log$_{10}$ 3.771/g. Total 29 enterobacterial isolates were isolated from the feed by using selected media. On the basis of morphological characteristics and biochemical test results the isolates were identified as Salmonella sp., Shigella sp., Klebsiella sp., Citrobacter sp., Proteus sp., Enterobacter sp. and Escherichia coli. These isolates were tested on blood agar medium and only seven isolates showed positive β-hemolytic activity. In virulence efficacy test, only hemolytic positive isolates were ingested to chicken and observed that Escherichia coli (SGE-1), Klebsiella sp. (SSE-6) and Salmonella sp. (JSS-9) isolates were highly toxic because the experimental chickens were died after 3 days of ingestion of the bacteria, two isolates showed loose motion symptom after 15 days while other isolates showed little sickness. All the selected isolates showed positive hem-agglutination reactivity in poultry RBC. The results indicate that the poultry feeds were highly contaminated with pathogenic enterobacteria which are risk to public health.

Key words: Enterobacteria, Hemagglutination, Hemolytic activity, Microbial quality, Poultry feeds

Introduction

Poultry is now a very important and widespread agricultural industry in the tropics. It is one of the major among livestock sub-sector that committed to supply cheap sources of good quality nutritious animal protein (20%) to the nation. In Bangladesh, there are 49,825 different types of poultry farms and out of them Rajshahi division belongs 20% and the expenditure for feed items was 41,091 million (BBS 2010). But the poultry disease remains one of the major threats to boosting poultry production. Enterobacteriaceae are a large group of related bacteria living in soil, water and decaying matter, and are also common occupants of both human and animal’s large bowel. They are acquired through contaminated food or water and are the major cause of enteric illnesses (Talaro and Talaro 2002). Microbiological risk factors can be found in all poultry production systems. The increasing problem of Salmonella infection is not necessarily attributable entirely to the growth and intensification of poultry production; changing consumption patterns may also be a

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factor. Other bacteria, such as *Clostridium perfringens*, *C. botulinum*, *Listeria monocytogenes* (Rorvik et al. 2006) and *E. coli* O157:H7 can also be found in poultry products (WHO 2007) and these organisms cause food-borne illnesses. Most of the pathogenic bacteria found in poultry meat are non–host-specific and are considered capable of causing human food poisoning. Besides pathogens associated with the animals themselves, organisms associated with humans, such as members of the enterobacteriaceae and *Staphylococcus* are major hygiene concerns in the handling of food products. Poultry and poultry meat are often found contaminated with potentially pathogenic microorganisms such as *Salmonella*, *Campylobacter*, *S. aureus*, *E. coli* and *Listeria*. In some occasions *Yersinia enterocolitica*, *Aeromonas* and *C. perfringens* have the potential to be important pathogens in poultry products. However, *Salmonella*, *Campylobacter* and to a lesser extent *Listeria*, are considered to be the major food-borne pathogens in the poultry industry (Hood et al. 1988). To meet the food security and public health, poultry production is increasing day by day and along with this the risk of food borne infection also occur frequently due to lack of unhygienic practices. In this situation Bangladesh is not out of risk for this problem but there is a very few research has done on hygienic practices and microbial safety in poultry feeds. Therefore, the present investigation has been undertaken to assess the microbial quality of poultry feeds through conventional culture method and virulence of some selected enterobacteria were also determined.

**Materials and Methods**

**Sample collection**

Poultry feed samples were collected from different commercial poultry farms of Rajshahi Metropolis and surrounding areas. The samples were collected into sterilized poly bags and transported to the Microbiology Laboratory, Department of Botany, and University of Rajshahi, Bangladesh and stored at 4°C for further processing. The samples were also coded properly according to the sources.

**Composition of the feed**

Only layer grower ration was used for microbial quality analysis and the feed components were as maize (48-52%), rice bran (16-22%), sesame oil cake (5-11%), soybean oil cake (9-11%), oyster shell (2-3%), wheat husk (5-10%), fishmil (8-12%), salt (50 g), premix L (500 g), lysene (250 g), metheonine (500 g), kolin (100 g) and larvadox (50 g).

**Sample processing and microbial analysis**

For microbial analysis, 1gm of poultry feed was vortex (VM-2000, rpm 300, Taiwan) with 90ml sterile distilled water to prepare homogenous mixture. Further tenfold serial dilution of the resultant homogenates was made up to 10⁶ dilution. From these dilutions, aliquots of 0.5 ml was inoculated in replicate plates of different media using the spread plate technique. Nutrient agar medium was used for total aerobic bacteria count, MacConkey agar for total coliform count, Eosine Methylene Blue (EMB) agar for total *Escherichia coli* count and SS- agar for total *Salmonella* and *Shigella* count. All the plates were incubated under aerobic conditions at 37°C for 24-72 hrs. The mean number of colonies counted was expressed as log colony forming units (cfu)/per gram.

**Isolation and identification of enterobacteria**

MacConkey, EMB and SS agar media were used for isolation of enterobacteria. Distinct colonies were isolated from the media on the basis of morphological variability. Pure culture of the isolates was obtained by streaking of a portion from the distinct isolated colonies on culture plate. For identification, morphological characteristics of the isolates on selective media were studied. Biochemical test of the isolates were done according to Bergey’s Manual of Determine Bacteriology. Motality, gram staining, indole, triple sugar iron,
methyl red, voges-proskauer (VP), citrate and catalase tests were done. Further identification was confirmed by using Micro-Rao online software.

**Hemolytic activity test**

Tryptose blood agar medium was used (5% beef blood) for determining the hemolytic reactions of the selected enterobacteria. The medium was poured into Petridis and loop full of each bacterial broth were streaked on blood agar separately and incubated at 37°C for 48 hrs. Then the plates were examined for growth and hemolytic reactions.

**Virulence test through direct ingestion**

Virulence test of selected isolates was carried according to Rat Pyometra Model (Mikamo et al. 1998) with slide modification. Each isolate was ingested with constant dose (MacForland OD 0.5) in poultry with three replications while sterile saline was ingested in control and observed for 15 days. Sickness and mortality of the poultry were monitored for each treatment. Percentages (%) of weight losses were determined following the under mentioned formula:

\[
\text{Percentage (\%)} \text{ of weight loss} = \frac{\text{Initial weight (W_i)} - \text{last weight (W_f)}}{\text{Initial weight (W_i)}} \times 100
\]

**Hemagglutination test**

Hemagglutination test of the selected isolates were performed following the method of Costabile (2010). Red blood cells from poultry were collected in 0.20 mM Tris-HCl buffer. 50 µl of 0.20 mM Tris-HCl buffer solution and 50 µl of each bacterial suspension was added in micro-titer plate according to A1-A4, B1-B4, C1-C4, D1-D4, and E1-E4, F1-F4 and G1-G4 no well and mixed properly. Then one-fold serial dilution of RBC was carried out as 1:1 dilution from wells no A1 to G1 down to A4 to G4. In case of control 50 µl of 20 mM Tris buffer were taken instead of bacterial suspension and 50 µl blood cell suspensions were mixed in H1 to H4 no well. The mixture in the titers plate was mixed well by gentle shaking with shaker continuously for 15 min and wait up to 30 min. After that, one drop of this suspension was examined under microscope for visible agglutination.

**Statistical analysis**

The experiment was conducted by using a completely randomized design with three replications. Statistical analysis (ANOVA) was performed using MS excel software (version-16.0.4266.1001). All data were reported as means with standard deviations.

**Results**

Microbial quality of poultry feed in different poultry farms of Rajshahi Metropolis were analysis on different media and the results are presented in Table 1 and Fig 1. In NA medium the ranges of cfu were counted as 2.8 × 10^5 to 5.8 × 10^9/g and 100% samples contained ≥10^6 cfu/g in total aerobic bacteria, while the ranges of total coliform were counted as 1.2 × 10^2 to 5.2 × 10^7/g in MacConkey agar and 50 to 100% samples contained ≥10^4 cfu/g. The ranges of cfu 1.03 × 10^2 to 1.09 × 10^5/g of E. coli were counted in EMB agar and 50 to 100% samples were contaminated with <10^2 cfu/gm. On the other hand, the ranges of cfu/g were counted as 1.02 × 10^1 to 5.25 × 10^4/g for total Salmonella in SS agar medium and 17 to 67% samples were contaminated with <10^2 cfu/g. The maximum mean bacteria were obtained as 8.797, 6.103, 4.493 and 3.665 log_{10} cfu/g from Iffat, Masum, Rakib and Raton poultry farm in NA, MacConkey, EMB and SS agar medium, respectively.
| Name of poultry farm and location | NA | MacConkey | EMB | SS |
|----------------------------------|----|------------|-----|----|
|                                 | % of sample >10^6 cfu/g (n = 6) | Ranges of cfu/g | % of sample >10^6 cfu/g (n = 6) | Ranges of cfu/g | % of sample >10^6 cfu/g (n = 6) | Ranges of cfu/g | % of sample >10^6 cfu/g (n = 6) |
| Ismail Poultry Farm, Court       | 83.33 | 1.2×10^6 to 4.4×10^6 | 1.03×10^5 to 1.48×10^5 | 50 | 1.02×10^3 to 4.85×10^3 | 16.66 |
| Mahbub Poultry Farm, Meherchondi | 100 | 1.4×10^6 to 3.2×10^6 | 1.04×10^5 to 3.96×10^5 | 66.66 | 2.26×10^3 to 4.13×10^3 | 50 |
| Juwel Poultry Farm, Chokpara     | 100 | 1.8×10^6 to 4.5×10^6 | 1.16×10^5 to 5.4×10^4 | 66.66 | 5.03×10^3 to 4.85×10^3 | 50 |
| Khokon Poultry Farm, Chormaajardiar | 100 | 2.3×10^6 to 2.5×10^6 | 3.17×10^6 to 5.12×10^6 | 66.66 | 6.09×10^3 to 1.54×10^6 | 50 |
| Mostak Poultry Farm, D asmari   | 100 | 1.1×10^6 to 0.7×10^6 | 1.09×10^5 to 3.65×10^4 | 66.66 | 5.01×10^3 to 4.25×10^4 | 50 |
| Belal Poultry Farm, Noudapara   | 100 | 1.6×10^6 to 6.5×10^5 | 4.30×10^5 to 5.23×10^5 | 66.66 | 3.11×10^5 to 1.20×10^5 | 66.66 |
| Raton Poultry Farm, Maherchondi  | 100 | 2.2×10^6 to 2.9×10^5 | 5.05×10^5 to 4.65×10^6 | 83.33 | 5.03×10^3 to 5.25×10^4 | 50 |
| Iflat Poultry Farm, Dangpara    | 100 | 1.3×10^6 to 2.5×10^5 | 1.08×10^6 to 3.22×10^6 | 83.33 | 1.05×10^5 to 1.20×10^5 | 50 |
| Sakil Poultry Farm, Parisal     | 100 | 1.4×10^6 to 4.5×10^4 | 1.09×10^6 to 1.70×10^5 | 83.33 | 5.02×10^3 to 5.11×10^5 | 50 |
| Rakb Poultry Farm, Daingpara    | 100 | 2.1×10^6 to 3.9×10^5 | 4.8×10^6 to 5.60×10^6 | 83.33 | 3.22×10^5 to 3.54×10^6 | 50 |
| Sohidul Poultry Farm, Katakhalii | 100 | 1.7×10^6 to 6.4×10^5 | 1.06×10^6 to 2.56×10^6 | 83.33 | 2.20×10^5 to 4.19×10^6 | 50 |
| Sojb Poultry Farm, Horipur      | 100 | 1.3×10^6 to 3.1×10^4 | 1.25×10^6 to 1.28×10^6 | 83.33 | 1.11×10^5 to 1.27×10^5 | 50 |
| Bulbul Poultry Farm, Haragram   | 100 | 4.3×10^6 to 2.5×10^5 | 1.12×10^6 to 2.35×10^6 | 66.66 | 1.14×10^5 to 1.21×10^5 | 50 |
| Masum Poultry Farm, Darusa       | 100 | 2.5×10^6 to 5.2×10^5 | 3.45×10^6 to 4.18×10^6 | 66.66 | 5.08×10^5 to 5.15×10^6 | 66.66 |

**Table 1.** Ranges of cfu of poultry feed samples on Nutrient agar (NA), MacConkey agar, Eosine Methylene Blue (EMB) agar and *Salmonella* and *Shigella* (SS) agar.
Fig. 1. Mean bacterial load of poultry feeds on different media of different poultry farms.
For isolation of enterobacteria, feed samples were placed on three selective media i.e. MacConkey agar, EMB agar and SS agar medium. Total 29 bacterial isolates were isolated from different media. The conformity level were detected as 99.9% for *Escherichia coli*, 98.41% for *Salmonella* sp., 91.81% for *Shigella* sp., 88.05% for *Citrobacter* sp., 100% for *Proteus* sp., 99.62% for *Enterobacter* sp., and 100% for *Klebsiella* sp. (Table 2). Further the isolates were subjected to pathogenicity test. In hemolytic test, out of 29 isolates, only 7 isolates showed β-hemolytic reactivity (Table 3 and Fig. 1). The virulence effect of these isolates was observed by ingestion of the selected bacterial isolates with standard dose in poultry model. Out of 7 isolates, SGE-1, SSE-6 and JSS-9 no. isolates were more virulent and caused death of chicken after 3 days of ingestion while isolates KSM-14 and AGM-22 showed loose motion symptom after 15 days and isolates ASS-12 and CSM-20 showed mild sickness (Table 4). All the isolates showed visual agglutination in red blood cell of poultry (Table 5 and Fig. 2).

**Table 2.** Biochemical test results of the selected isolates of enterobacteria

| Isolate codes | OX | IN | MO | CA | CI | PH | MR | VP | GL | LC | SC | TSI | Suspected bacteria (% of conformity) |
|---------------|----|----|----|----|----|----|----|----|----|----|----|-----|-------------------------------------|
| JSS-9, SGS-11, ASS-12, MLS-13, RGS-8, MGS-10, SGN-25, MLN-27, SSE-6, IGM-15, BSN-24, SSM-18, ISM-19, RSM-21 | -  | -  | +  | +  | -  | +  | -  | -  | +  | +  | +  | R   | Y   | +  \(^{Salmonella\ sp. (98.41\%)}\) |
| AGE-7, KSM-14 | +  | +  | +  | +  | -  | +  | -  | +  | +  | +  | +  | Y   | Y   | -  \(^{Shigella\ sp. (91.81\%)}\) |
| ASN-26, MGN-28, AGM-22 | -  | -  | +  | +  | -  | +  | +  | +  | +  | +  | +  | R   | Y   | +  \(^{Citrobacter\ sp. (88.05\%)}\) |
| ISE-3, RGN-23, CSM-20, ISN-29, SGE-1, JSE-2, MLE-4, MSE-5, BGM-16, MGM-17 | -  | +  | +  | -  | -  | +  | +  | +  | +  | +  | +  | Y   | Y   | -  \(^{Enterobactersp. (99.62\%)}\) |

OX = Oxidase, IN = Indole, MO = Motility, CA = Catalase, SH = Starch hydrolysis, CI= Citrate, PH = Phenylalanine, MR = Methyle red, VP = Voges-Proskauer, TSI = Triple Sugar Iron, H₂S = Hydrogen sulphide, GL = glucose, LC = Lactose, SC = Sucrose, R = Red, Y = Yellow, + = Positive,- = Negative
Table 3. Hemolytic activity test results of isolated bacteria

| Sl. No. | Code of isolates | Hemolytic activity | Sl. No. | Code of isolates | Hemolytic activity |
|---------|------------------|--------------------|---------|------------------|--------------------|
| 1       | SGE-1            | +++                | 16      | BGM-16           | ++                 |
| 2       | JSE-2            | ++                 | 17      | MGM-17           | ++                 |
| 3       | ISE-3            | ++                 | 18      | SSM-18           | +                  |
| 4       | MLE-4            | ++                 | 19      | LSM-19           | -                  |
| 5       | MSE-5            | ++                 | 20      | CSM-20           | +++                |
| 6       | SSE-6            | +++                | 21      | RSM-21           | ++                 |
| 7       | AGE-7            | +                  | 22      | AGM-22           | +++                |
| 8       | RGS-8            | -                  | 23      | RGM-23           | +                  |
| 9       | JSS-9            | +++                | 24      | BSN-24           | +                  |
| 10      | MGS-10           | ++                 | 25      | SGN-25           | -                  |
| 11      | SGS-11           | +                  | 26      | ASN-26           | ++                 |
| 12      | ASS-12           | +++                | 27      | MLN-27           | +                  |
| 13      | MLS-13           | +                  | 28      | MGN-28           | +                  |
| 14      | KSM-14           | +++                | 29      | ISN-29           | +                  |
| 15      | IGM-15           | +                  |         |                  |                    |

+++ = β hemolysis, ++ = smaller clear zone, + = very small clear zone, - = no hemolysis.

Table 4. Toxicity test results of selected enterobacteria on chicken

| Isolates code | Constant dose 1.5 x 10^8/ml | Initial weight (g) | Weight loss (g) after different days (d) | % of Weight loss | Symptom         |
|---------------|-----------------------------|--------------------|------------------------------------------|-----------------|-----------------|
|               |                             |                    | 3 d | 6 d | 9 d | 12 d | 15 d |                      |                 |
| SGE-1         | +                           | 645±2.8            | 632±1.4 | - | - | - | - | 2.02 | Dead               |
| SSE-6         | +                           | 635±1.1            | 619±1.8 | - | - | - | - | 2.52 | Dead               |
| JSS-9         | +                           | 475±4.5            | 461±3.2 | - | - | - | - | 2.94 | Dead               |
| ASS-12        | +                           | 605±1.4            | 590±2.8 | 570±1.1 | 545±1.2 | 530±0.7 | 520±1.41 | 8.93 | Little sick        |
| KSM-14        | +                           | 480±4.9            | 472±3.3 | 457±2.8 | 446±4.9 | 418±1.06 | 395±2.83 | 17.70 | Loose motion       |
| CSM-20        | +                           | 520±0.8            | 500±3.3 | 480±1.1 | 465±4.5 | 450±1.25 | 440±1.41 | 10.19 | Little sick        |
| AGM-22        | +                           | 658±2.2            | 655±3.8 | 648±1.4 | 641±2.8 | 636±4.95 | 632±2.83 | 3.95  | Loose motion       |
| Control       | -                           | 635±3.8            | 654±1.7 | 665±2.8 | 672±4.1 | 683±0.7 | 690±1.41 | Nill          | Healthy           |
Table 5. Hemagglutination test results in chicken blood

| Organisms                      | Hemagglutination activity |
|--------------------------------|----------------------------|
| *E. coli* (SGE-1)             | +                         |
| *Klebsiella* sp. (SSE-6)      | +                         |
| *Salmonella* sp. (JSS-9)      | +                         |
| *Salmonella* sp. (ASS-12)     | +                         |
| *Citrobacter* sp. (KSM-14)    | +                         |
| *Enterobactor* sp. (CSM-20)   | +                         |
| *Proteus* (AGM-22)            | +                         |

+ = positive agglutination.

Fig. 2. Photographs showing pathogenicity test results: No hemolysis (A), β-Hemolysis (B), agglutination occurred in micro-titer plate (C), and microscopic view of positive hemagglutination (D).
Discussion

Poultry feed are the media where microorganisms can grow easily during production, processing and storage. This study was performed to assess the bacterial load of poultry feeds samples for detection of sanitary level of the feeds. From the results it reveals that poultry feeds in Rajshahi were highly contaminated with aerobic bacteria and hundred percent samples crossed the limit of international standard for microbial safety (Hood et al. 1988). Large number of coliforms were recorded in poultry feed samples and seventy five percent samples remained in microbial hazard condition. The moderate number of *E. coli* was recorded in the feed samples and about seventy percent samples were exceeding the international standard. The small number of *Salmonella* sp. were also observed in poultry feed. From the results it was exhibited that all the feed samples of the poultry farms of Rajshahi Metropolis were highly contaminated with coliforms and others bacteria. In earlier study De-Shalom (1999) investigated the bacterial contaminants associated with commercial poultry feeds and reported *Staphylococcus aureus* as the most predominant bacterial organism with 52 cfu/g, followed by *Salmonella typhi* with 48 cfu/g, *Bacillus cereus* 40 cfu/g and *Pseudomonas aeruginosa* 18 cfu/g. The findings also concurred with other study which gave total plate count, total coliforms and *E. coli* as log$_{10}$ 4.99/g, 4.49 log$_{10}$ cfu/g and <3.85 log$_{10}$cfu/g (Higenyi et al. 2014).

Enterobacteria are bacteria from the family Enterobacteriaceae, which are primarily known for their ability to cause intestinal upset. Enterobacteria are responsible for a variety of human illnesses, including urinary tract infections, wound infections, gastroenteritis, meningitis, septicemia, and pneumonia. Some are true intestinal pathogens; whereas others are merely opportunistic pests which attack weakened victims. Total 29 enterobacterial isolates were isolated which were identified as *Salmonella* sp., *Shigella* sp., *Klebsiella* sp., *Citrobacter* sp., *Proteus* sp., *Enterobacter* sp. and *Escherichia coli*. In earlier study Ahmed (2010) identified *E. coli*, *Klebsiella* sp, *Proteus vulgaris*, *Hafnia alive*, *Salmonella* sp. from poultry feed in Khartoum state which supports the findings of present research.

A potential and more deadly hazard has been associated with the consumption of microbial toxins of bacterial and fungal origin in feed (Gilbert 1995). On the other hand, presence of *E. coli* and *Salmonella* spp. may suggest fecal as well as environmental contamination (Uwaezuoke and Ogbulie 2008). For instance *E. coli* known as coliform bacteria are normal inhabitants of the digestive tract and are abundant in the poultry environment, some of them is implicated in disease conditions such as colibacillosis occurring various forms such as enteric and septicemic colibacillosis that cause increased mortality and performance of birds. *Salmonella* spp. also a serious threat to consumer health due to its ability to adapt to many different environments and broad range of transmission routes producing acute and chronic infections in all or most types of birds and animals (Barnes et al. 2003, Maciorowski et al. 2004). Pathogenic potentiality of the selected isolates were tested and out of twenty-nine isolates, seven isolates showed β-hemolytic activity on blood agar because these isolates break down red blood cells and resulting a clear zone were formed surrounding the colony. Isenberg (1992) reported the similar findings. For virulence test seven hemolytic isolates were selected and standard dose of these isolates were directly ingested to healthy chicken. Out of seven isolates, *E. coli* (SGE-1), *Klebsiella* sp. (SSE-6) and *Salmonella* sp. (JSS-9) isolates showed highly toxic reactivity and the chickens were died after 3 days while two isolates showed loose motion symptom after 15 days and other isolates showed mild sickness compare to control. Similar experiment was conducted by Rahman (2009) while observed positive results of hemolytic test and pathogenecity test for *Escherichia coli, Salmonella* sp., *Klebsiella* sp., *Staphylococcus* sp. and *Bacillus* sp. in mice. In another study *Salmonella* produced clinical sign in poultry due to lower dose (3.15 × 10⁴) of ingestion of the organism (Wray et al. 1996). Hemagglutination assay was performed using chicken RBC and all the isolates showed visual positive agglutination results. From the results it may concluded that the commercial poultry feed used in the farms of Rajshahi Metropolis where not in safe condition. Not only that the feed also highly
contaminated with potential pathogenic bacteria like *Escherichia coli*, *Salmonella* sp., *Klebsiella* sp. and *Proteus* sp. which may play adverse effect on poultry farming and public health.

**Acknowledgement**

The authors are thankful to the University Grant Commission (5/52/UGC/LifeScience-03/16-17/60, date-18/12/16) for the financial support of this research.

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