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The occurrence of drug-resistant bacteria and screening the possible presence of residual antibiotics in poultry feed samples

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The use of antibiotics in the poultry and livestock industries for the treatment and prevention of infectious diseases, and as growth promoters in poultry feeds has increased worldwide. Such frequent employment of antibiotics may contribute to the development and dissemination of bacterial antibiotic resistance. The present study was an attempt to isolate drug-resistant bacteria and to screen the probability of having residual antibiotics in the poultry feed samples. Therefore, a total of 18 samples inclusive of starter, grower and finisher of two poultry feed brands of reputed Bangladeshi feed companies were collected and subjected to microbiological analysis, antibiogram and agar well diffusion assay. All the samples contained extended numbers of total viable bacteria and fungi in an average of 10^8 and 10^7 cfu/g, respectively. *Klebsiella* spp., *Pseudomonas* spp. and *Bacillus* spp. were predominantly present in the tested samples. *E. coli* and *Vibrio* spp. were also found in most of the samples. Most isolates have been determined to be multidrug-resistant. All the isolates showed resistance against Cefuroxime. Penicillin resistance was found in most of the isolates in greater proportion. Higher rate of resistance was evident against Novobiocin, Cephradine and Rifampicin. However, the bacterial isolates showed sensitivity to Tobramycin, Nalidixic acid and Neomycin. The poultry feed samples, especially starter and finisher of both brands noticeably had significant antimicrobial activity against the laboratory isolates indicative of the probable presence of residual antibiotics which might be used as supplements in the poultry feed samples.

Keywords: Poultry feed, Microbiological analysis, Drug resistance, Residual antibiotics.

INTRODUCTION

Poultry refers to all man-domesticated birds including chicken, duck, turkey, pigeons, quail etc. serving as an economically profitable resource of high quality protein for human consumption worldwide (1-3). Poultry feeds are formulated to fulfil the diverse nutritional requirements of birds. Since birds have the simple digestive tract and the intestinal flora merely affects the digestion process, there is a sharp need for easily digestible poultry feed, which is important for accelerating growth and egg production (1, 4). Feed materials are obtained from animals and plants of various origins and are primarily agro-wastes (4, 5). Poultry feed comprises mainly grains such as maize, wheat, barley, cake meal, sunflower seeds, peanuts and animal protein products such as fish meal, meat or bone meal, etc. (4, 6, 7). Depending on the functions they perform in the birds, different categories of poultry feeds exist inclusive of starter, growers, finishers, layers etc. (7).

Nevertheless, most of these feed components are one of the main sources of microbial intervention which may be of public health concern (3). Microorganisms residing in the feed may be their normal flora or result of cross-contamination (8). Dirt, dust, rodent, birds, human carrier, sewage or water during processing and storage can transmit microbial commodities in poultry feed (9). Major feed contaminants include moulds, mycotoxins and bacteria which may be responsible for various poultry diseases like cholera, colibacillosis, listeriosis, staphylococcosis, amoebic dysentery, bacillary dysentery, salmonellosis, avian influenza, newcastle disease, infectious coryza etc. (1, 3, 7, 10). *Salmonella* spp. and other bacterial pathogens those are contaminating poultry products can spread to human via the food chain (11). The emergence of drug-resistant bacteria in feed and feed ingredients may increase the risk associated with foodborne diseases (2).

In poultry feeds, a large diversity of antimicrobials including bambermycin, bacitracin, salinomycin, penicillin is used to encourage growth and prevent infectious diseases in poultry (12, 13). These antibiotics probably accelerate feed conversion and body weight gain by altering the microflora (14). This can modulate the intestinal flora and generate a selective pressure for resistant bacteria (13, 14). Also, the indiscriminate use of such important antimicrobials in the poultry industry is likely to accelerate the development of antibiotic resistance in both pathogens and commensal species. In addition, the presence of antimicrobial residues in poultry products (meat and eggs) also increases human health concerns due to antibiotic resistance (15).

Considering these facts, the present study was attempted to screen the presence of drug-resistant

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microorganisms in the poultry feed samples. The current research also investigated the antibacterial activity of poultry feed samples which would be indicative of the presence of antibiotic residues in the feed samples.

MATERIALS AND METHODS

Sampling and sample processing. Three samples each of starter (used to feed 0-12 days old chickens), grower (used for 13-25 days old chickens) and finisher (used to feed ≥26 days old chicken until sold) feed samples of two reputed brands of Bangladesh were collected in the sterile jar from a poultry farm located in Pabna town and transported to the laboratory at the earliest convenient. All the samples were prepared, processed and analyzed during the period between January, 2019 to April, 2019 in Microbiology Laboratory of Stamford University Bangladesh. For the identification and enumeration of bacteria and fungi, 10g of each sample was blended with 90 ml of normal saline and diluted up to 10^{-6} according to the standard guideline (16-22).

Microbiological Analysis. An aliquot of 0.1 ml of each sample from the dilutions 10^{-5} and 10^{-6} was inoculated onto the Nutrient Agar (NA) and Sabouraud's Dextrose Agar (SDA) plates employing spread plate technique to isolate total viable bacteria (TVB) and fungi, respectively (16-22). Similarly, the isolation and enumeration of total coliforms (especially, *Escherichia coli* and *Klebsiella* spp.), fecal coliforms, *Staphylococcus* spp., *Bacillus* spp., *Pseudomonas* spp., *Vibrio* spp. and *Salmonella* spp. was carried out by spreading 0.1 ml of each sample from the dilutions 10^{-3} and 10^{-4} onto the MacConkey agar, Membrane Fecal Coliform (mFC) agar, Mannitol Salt Agar (MSA), Mannitol Yolk Polymyxin (MYP) agar, *Pseudomonas* agar, Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar and *Salmonella-Shigella* (SS) agar, respectively. Plates for bacterial growth were incubated at 37°C for 24 h. SDA agar plates for the growth of fungi and mFC agar plates for fecal coliform were held at 25°C for 48 h and at 44.5°C for 24 h, respectively (16-22).

Antibiotic susceptibility test of the isolates by agar-disc diffusion method. The standard agar-disc diffusion method (Kirby-Bauer technique) was employed to determine the antibiotic susceptibility of the isolates (either sensitive or resistance) on Mueller-Hinton agar (Difco, Detroit, MI) (17-19, 23, 24). The commercially available antibiotic discs tested in this experiment were Tobramycin (TOB, 10 µg), Novobiocin (NV, 30 µg), Penicillin (PEN, 10 µg), Cephradine (CE, 30 µg), Nalidixic acid (NA, 30 µg), Cefuroxime (CXM, 30 µg), Cefixime (CFM, 5 µg), Rifampicin (RIF, 5 µg), Erythromycin (E, 15 µg) and Neomycin (N, 30 µg). After placing the discs, the MHA plates were inverted and incubated at 37°C for 24 h. After incubation, the plates were examined for measuring the zone of inhibition in mm.

Assessing the presence of residual antibiotics in poultry feed samples through agar well diffusion method. For the evaluation of antimicrobial activity indicative of the presence of residual antibiotics, modified agar well diffusion method was applied using Mueller-Hinton agar plate (17, 18, 21, 22). Suspension of previously isolated and stored laboratory bacterial species such as *E. coli*, *Pseudomonas* spp., *Vibrio* spp., *Klebsiella* spp., *Staphylococcus aureus* and *Salmonella* spp., were prepared using normal saline, consisting of 10^5 cfu/ml with turbidity equivalent to that of the 0.5 ml McFarland standard. Afterwards, each suspension was subjected to lawn on the Muller-Hinton agar (MHA) (Oxoid Ltd., Basingstoke, Hampshire, England). The wells were dug (8 mm^3) on the inoculated Muller Hinton agar Medium, and 100 µl of 11mg/ml of each homogenized poultry feed sample was dispensed into the well. Normal saline was used as a negative control whereas antibiotic disc of Gentamycin (GEN, 10 µg) was used as positive control. The plates were incubated at 37°C overnight and examined for the zone of inhibition. The diameter of the inhibition zone was measured in mm using slide callipers.

RESULTS AND DISCUSSION

Microbiological condition of the tested poultry feed samples and the recovery of antibiotic-resistant bacteria. Different study findings stated that poultry feeds are one of the major sources of microorganisms to poultry products (3, 25). As proof of this statement, all the samples in the present study were found to contain a huge array of microorganisms irrespective of the brands (Table 1). Total viable bacteria and fungi were encountered at an average of 10^8 cfu/g and 10^7 cfu/g, respectively. Such high fungal and bacterial load may correspond to a potential hazard to the birds (3). Among the specific pathogenic bacteria, *Klebsiella* spp., *Pseudomonas* spp. and *Bacillus* spp. were predominant as found in every sample in an average of 10^5 cfu/g. *Vibrio* spp. were also found in almost every sample expect grower of brand 1 (Table 1). *E. coli* were only absent in finisher of brand 1 and starter of brand 2. Grower and finisher of brand 2 were found to contain *Staphylococcus* spp. *Salmonella* spp. were encountered in starter of brand 1 and finisher of brand 2. All the samples were devoid of the presence of fecal coliforms (Table 1). Previous studies in different parts of the world also reported the presence of various microorganisms in poultry feed that can lead to food-borne infections (1, 7, 8, 11, 26-28). As experimented and stated by Crumps et al. (11), Shirota et al. (29) and Hald et al. (30), poultry feeds are key sources of *Salmonella* and other bacteria transmission in the poultry industries. During feed preparation, physical and chemical treatments should be implemented to minimize the bacterial load for the

Table 1. Microbiological load in the tested poultry feed samples.

Poultry feed Sample (n=18)	Microbial load (cfu/g)								
	TVB	**TFC**	***E. coli***	***Kebsiella spp.***	***Pseudomonas* spp.**	***Bacillus* spp.**	***Staphylococcus* spp.**	***Vibrio* spp.**	***Salmonella* spp.**
Brand 1 (n=9)									
Starter (n=3)	2.8×10^8	3.5×10^7	4.1×10^5	1.8×10^6	7.5×10^5	5.0×10^5	0	1.5×10^5	5.5×10^5
Grower (n=3)	3.6×10^8	2.5×10^7	2.0×10^4	7.5×10^5	1.4×10^5	1.2×10^5	0	0	0
Finisher (n=3)	3.2×10^8	6.0×10^7	0	5.5×10^5	5.0×10^4	4.0×10^5	0	3.0×10^4	0
Brand 2 (n=9)									
Starter (n=3)	2.6×10^8	3.8×10^7	0	1.8×10^5	3.0×10^4	1.6×10^5	0	6×10^4	0
Grower (n=3)	1.2×10^8	4.0×10^7	8.0×10^4	1.1×10^5	1.1×10^6	2.5×10^6	2.5×10^5	2.3×10^6	0
Finisher (n=3)	2.0×10^8	3.6×10^7	3.5×10^5	2.5×10^5	3.5×10^5	6×10^5	5.0×10^5	5.5×10^5	1.5×10^5

Note: TVB = Total viable bacteria; TFC = Total fungal count.
The experiments were in triplicates. Mean count (cfu/g) from all samples have been shown here.
Fecal coliforms were absent in all samples.

Table 2. Antibiotics susceptibility pattern of the bacterial species isolated from the poultry feed samples.

Bacterial Isolate	Antibiotic discs									
	TOB	**NV**	**PEN**	**CE**	**NA**	**CXM**	**CFM**	**RIF**	**E**	**N**
Brand 1										
E. coli	S	R	R	R	S	R	S	R	R	S
Klebsiella spp.	S	R	R	R	S	R	R	R	S	S
Vibrio spp.	S	R	R	R	S	R	S	R	S	S
Salmonella spp.	S	R	R	R	S	R	R	R	R	S
Pseudomonas spp.	S	S	R	S	S	R	S	S	S	S
Staphylococcus spp.	S	S	R	S	S	R	S	S	S	S
Bacillus spp.	S	S	R	S	S	R	S	S	S	S
Brand 2										
E. coli	S	R	R	R	S	R	S	R	S	S
Klebsiella spp.	S	R	R	S	S	R	S	R	R	S
Vibrio spp.	S	R	R	R	S	R	S	R	S	S
Salmonella spp.	S	R	R	R	S	R	R	R	R	S
Pseudomonas spp.	S	R	R	R	S	R	S	R	R	S
Staphylococcus spp.	S	S	S	S	S	R	S	S	S	S
Bacillus spp.	S	R	R	R	S	R	S	S	S	S

Note: R = Resistant; S = Sensitive; TOB = Tobramycin (10 µg); NV = Novobiocin (30 µg); PEN = Penicillin (10 µg); CE = Cephradine (30 µg); NA = Nalidixic acid (30 µg); CXM = Cefuroxime (30 µg), CFM = Cefixime (5 µg), RIF = Rifampicin (5 µg); E = Erythromycin (15 µg); N = Neomycin (30 µg).

Table 3. Antimicrobial effects imparted by the poultry feed samples.

Poultry feed samples	Zone of inhibition (mm) against tested microorganisms					
	E. coli	***Klebsiella* spp.**	***Pseudomonas* spp.**	***Staphylococcus* spp.**	***Vibrio* spp.**	***Salmonella* spp.**
Brand 1						
Starter	7 mm	0	9 mm	7 mm	8 mm	16 mm
Grower	0	0	0	0	0	0
Finisher	14 mm	10 mm	17 mm	0	0	0
Brand 2						
Starter	6 mm	7 mm	8 mm	0	0	8 mm
Grower	0	0	0	0	0	7 mm
Finisher	12 mm	0	7 mm	8 mm	0	0

better quality product and storage conditions, packaging, handling should be aseptically maintained as well (1).

If antibiotics in poultry feeds are used for growth promotion purposes, a limited quantity is always provided in contrast to therapeutic usage. This can contribute to antibiotic resistance by bacteria (31). Mahami et al. (32), Sule and Ilori (33), and Donkor et al. (34) reported the presence of multi-drug resistant bacteria in their study on poultry feeds. In the present study, All the isolates from the samples of brand 1 and brand 2 showed their resistance against multiple drugs in cohort with those studies (Table 2). In cases of isolates from brand 1, Penicillin and Cefuroxime resistance were found in all the isolates. Whereas, the isolates showed sensitivity against Tobramycin, Nalidixic acid and Neomycin. Cephradine, Novobiocin and Rifampicin resistance were found in *E. coli*, *Klebsiella* spp., *Vibrio* spp. and *Salmonella* spp. *E. coli* and *Salmonella* spp. exhibited resistance against Erythromycin. Cefixime resistance was found in *Klebsiella* spp. and *Salmonella* spp (Table 2). On the other hand, the isolates of brand 2 in cohort with that of brand 1 showed sensitivity against Tobramycin, Nalidixic acid and Neomycin (Table 2). Similarly, all the isolates of brand 2 exhibited resistance against Cefuroxime. Penicillin and Novobiocin resistance were found against almost all the isolates except *Staphylococcus* spp. Only *Salmonella* spp. showed resistance against cefixime (Table 2). In this study, the recovery of rather higher multidrug resistant bacterial strains in the poultry feed samples could have resulted from the misuse and overuse of antibiotics which indicates the lack of regulation and proper policies in the agriculture sector, especially in the developing countries (32).

The emergence and spread of antibiotic resistance will potentially hamper the nutritional and economic prospects of poultry (31).

Determination of the possible presence of residual antibiotics in poultry feed samples. The use of antibiotics has successfully and economically improved poultry efficiency, but the resulted increase in the number of antibiotic-resistant strains that can be transmitted through the food chain from poultry to humans can have serious public health consequences (1). Multidrug resistance arises in bacteria due to the accumulation of resistance genes from other bacteria, especially when they expose to an environment containing antibiotic residues (32, 35). Hence, the current study attempted to tract the presence of antibiotic residues in the feed samples by agar well diffusion technique. In the present study, the poultry feed samples, especially the starter and finisher of both the brand exhibited noticeable antibacterial traits against tested laboratory strains which was the indication of the probable presence of residual antibiotics in the feed samples (Table 3). The starter of brand 1 had an inhibitory effect on all the bacteria except *Klebsiella* spp. with the larger zone of inhibition (16 mm) against *Salmonella* spp. The finisher samples had antimicrobial activity against *E. coli*, *Klebsiella* spp. and *Pseudomonas* spp. with the significant zone of inhibition. Grower of brand 1 had no suppressing effect on any of the bacterial isolates (Table 3). On the other hand, grower of brand 2 had antimicrobial activity only against *Salmonella* spp. *E. coli*, *Klebsiella* spp., *Pseudomonas* spp. and *Salmonella* spp. were found to be slightly inhibited by starter of brand 2. Finisher of brand 2 had an inhibitory effect on *E. coli*, *Pseudomonas* spp. and *Staphylococcus* spp. (Table 3). The presence of antimicrobial traits in the poultry feed samples projects the nondiscriminatory use of antibiotics for feed production as stated in their study by Mahami et al. (32).

CONCLUSION

The findings of the present study have shown a relatively high level of microbial contamination in poultry feed which is of public health concern posing threat to both animal and human. Routine microbiological examinations of poultry feed are therefore imperative to increase poultry quality and production efficiency. Recovery of drug-resistant bacteria and the presence of significant antibacterial traits in the poultry feed samples suggests the urgent need to increase public and governmental concerns in eliminating sub-therapeutic use of antibiotics in poultry and livestock, especially those antimicrobials that are also used to treat humans. Besides, alternatives to antibiotics such as the application of probiotics in poultry should critically be considered for the production of safe edible products.

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