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Prevalence of microflora in trachea of NDV challenged broiler following supplementation with extracts of *Aloe vera, Alma millsoni, Archachantina marginata and Ganoderma lucidum*

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Abstract
An attempt was made to enumerate the micro-flora in trachea of broiler chickens challenged with Newcastle disease virus (NDV) following supplementation with different extracts of local sources. After 30 days of treatment with extracts of *Aloe vera* (AV), *Alma millsoni* (*ALM*), *Archachatina marginata* (AM) and *G. lucidium* (GL) *ad libitum* as supplement to various groups they were challenged with intramuscular administration of 0.2 ml of 10^6 ELD_{50} of NDV. Following clinical signs and symptoms in the birds, trachea swab samples were collected and cultured. Of the 95 swabs, 65 (68.4%) yielded mixed growth of bacteria spp. *Pseudomonas aeruginosa* (23.1%), *Enterococcus feacalis* (23.1%) and *Staphylococcus spp.* (23.1%), coliform bacteria (30.8%). Only 9 (13.9%) of the total population isolated resisted either cerofloxacin or genticin. There was no statistically significant difference ($P<0.05$) in the occurrences of bacteria in trachea of chickens supplemented with different concentration of extracts, both challenged with NDV and unchallenged groups. These organisms may not be pathogenic to the birds as there were no signs of bacterial infection on the trachea. Though, 86.1% of the isolates were sensitive to test drugs, evolution of strains resistant to common antibiotics by some of these bacteria could be of public health risk to handlers and the community. Thus, domestic birds keeping and poultry sited in close proximity to human dwellings should be outlawed.
Key words: Bacteria, Bacteria isolation, Broilers**,** Resistance

1.0 Introduction

The routine handling of animals entails certain personal risks. Steps must be taken to protect the handler. Training is the best way to learn how to handle a bird without injuring the bird or running the risk of a bite. Any wild animal, even if not aggressive, may attack with painful if not serious results. A variety of diseases are transmittable from birds to humans (Evans and Carey, 1986; Abu*ALM*eesh *et al*., 2006). Common among these are campylobacteriosis, histoplasmosis, ornithosis, tuberculosis, salmonellosis and *Yersinia spp.* (enterocolitia and pseudotuberculosis) as well as tick - borne diseases.

The evolution of antibiotic resistance to common antibiotics by some of these bacteria, as a result of heavy usage of antibiotics to enhance livestock production performance has been documented. In a study of poultry and farm personnel, Ojeniyi (1989) reported increase antibiotic resistance in commercial reared birds compared to free range village poultry. The same study reported that similar resistance patterns were isolated among poultry personnel and birds but not in village controls. Silbergeld *et al*. (2008) buttressed the point in their reported, that agricultural antimicrobial drug use was a major driver of antimicrobial resistance worldwide for four reasons: It is the largest use of antimicrobials worldwide; much of the use of antimicrobials in agriculture results in sub therapeutic exposures of bacteria; drugs of every important clinical class are utilized in agriculture; and human populations are exposed to antimicrobial-resistant pathogens via consumption of animal products as well as through widespread release into the environment.

Bacteria resistance to antibiotic is of great concern in the medical community. Large amounts of antibiotics used for human therapy, as well as for farm animals and even for fish in aquaculture, resulted in the selection of pathogenic bacteria resistant to multiple drugs (Nikaido, 2009). Sometimes the insertion of foreign DNA by recombination accomplishes the same end. More often resistance genes are carried on extra chromosomal plasmids that may be transferable from organism to organism by conjugation, transduction, or transformation (Jacoby and Swartz, 1980). Moreover, there is also a growing need for new agents against community-acquired pathogens, including the agents of tuberculosis, gonorrhoea and urinary tract infections (Livermore, 2004). Suffice to say that the likely consequences of such occupational hazard to poultry bird handlers may be enormous. Felix Marti-Ibanez, 1955 cited by Harbarth and Samore (2005) stated that antibiotic therapy, if indiscriminately used, may turn out to be a medicinal flood that

temporarily cleans and heals, but ultimately destroys life itself." Thus, the enumeration of micro-flora in trachea of broiler chickens challenged with NDV and their susceptibility to commonly used antibiotics.

2.0 Material and methods

2.1 Laboratory animals (broilers)

The experimental animals comprised of 380-day-old broilers obtained from Obasanjo Hatchery, Oluyole Estate, Ibadan, Oyo State. They were transported to Animal House, Achievers University, Owo, Ondo State, Nigeria.

2.2 Innoculums

A vial of lyophilized challenged strain of Newcastle disease virus (NDV) was obtained from Regional Laboratory for Avian Influenza and Tran boundary Animal Diseases, National Veterinary Research Institute, Vom. The vial was transported under cold chain and standard bio-safety practice to Owo, Ondo State. A saline suspension of 10^6 ELD_{50} was prepared by taken up the vial in 1.5 ml of sterile diluents (physiological saline), then take 1ml of the reconstituted virus to add 99.0 ml sterile normal saline.

2.3 Bacteria

A pure culture of *Staphylococcus aureus* (NCTC 6571) and *Escherichia coli* (NCTC 10418) were collected from the Nigerian Institute of Medical Research (NIMR) Yaba Lagos. They were confirmed using standard Microbiological methods (Cheesbrough, 2006). They were used for the standardizing of the zone of inhibition of bacteria isolates from the tested birds.

2.4 *Alma millsoni* (*ALM*) extracts

Samples of *Alma millsoni* was sourced from riverside of Okitipupa, Ondo State, washed with rain water and transported in a clean plastic bucket with sand and water to the laboratory for processing. Samples deposited in Zoology Laboratory, Federal University of Agriculture, Abeokuta were identified and authenticated by Dr. A. A. Aladesida. The *millsoni* tonic extraction was carried out according to the method described by Ang Lopez and Raelm (2006).

2.5 *Aloe vera* (AV) extracts

Samples of the succulent leaves of aloe plant were harvested and washed with distilled water. Samples deposited in Biological Science Department, Achievers University, Owo, were identified by Prof. S. O. Oyewole. *Aloe vera* juice was prepared following the method described by Wu *et al.* (2006) with slight modification. The modification briefly stated included the following steps: Freshly harvested leaves of the plant were washed, drained and cut open. The inner pulp was scrapped into a clean beaker and warmed at 50^{o}C for 30 min. until the viscous light - yellow pulp became less viscous. The extract was filtered with a muslin cloth.

2.6 *Ganoderma lucidum* (GL) extracts

Fruiting bodies of *G. lucidum* were obtained locally from open forest at Ipele in Ose Local Government Area of Ondo State, Nigeria. The fungal material was identified by Dr. S. Fakoya of the Department of Biological Sciences, Joseph Ayo Babalola University, Ikeji-Arakeji, Osun State, Nigeria, where voucher specimen number 1103 has been deposited. Ganoderma extract was prepared using aqueous extraction method as described by Oluba *et al*., 2010.

2.7 *Archachatina marginata* (AM) extracts

The homemade giant land snails were transported to laboratory for processing. Samples deposited in Zoology Laboratory, Federal University of Agriculture, Abeokuta were identified and authenticated by Dr. A. A. Aladesida. The snails were washed thoroughly and rinsed with distilled water. With the aid of giant forceps, the shell were carefully cracked to access the sexual apparatus and tapped out juice/fluid from the albumin gland into beaker. The fluid was then centrifuged at 5000 rpm for 10 min. thereby yielding supernatant, precipitated and gelatinous sludge. The bluish supernatant was carefully extracted into conical flask.

2.8 Preparation of extracts

The extracts of AV, *ALM*, AM and GL were evaporated to dryness at 37℃ using a Speed Vac (Model 7811001, Labconco, USA). The recovered extract was weighed and formulated in distilled water to give the required dose.

2.9 Treatment of broilers

During the entire study the broilers were fed with feeds compounded carefully to meet 23% crude protein (CP) and 3200 K.cal. metabolizable energy (ME) for broiler starter and 20% CP and 3000 K.cal. for broiler finisher. We ensured that the levels of mycotoxins in feed were maintained relatively low throughout the experiment, the quality and quantities of groundnut cake (GNC), soya bean and rice bran included in the feeds were the same for both starter and finisher mash. The percentage CP in feed and ingredients were determined by the biurette method (Ranjna, 1999) while the metabolizable energy was determined by the Bomb calorimeter method (AOAC, 1980).

The broilers were housed in battery cages 0.31 m^2 / bird as recommended by Mustafa *et al*. (2010). All experimental protocols complied with NIH guidelines (NRC, 1985), as approved by the ethical and research committee, Achievers University, Owo. All the birds received necessary medication and vaccination exempting NDV vaccine with the exception of the NDV vaccinated control group. The birds were divided into 19 groups of 20 birds each; they were allowed to acclimatize for 16 days and were fed with standard broilers feed and water *ad libitum.* The group A were supplemented with *Aloe vera* extract, group B were supplemented with *Alma millsoni* (earthworm) extract, group C were supplemented with *Archachatina marginata* (snail serum) and group D were supplemented with *Ganoderma lucidum* (Lingzhi) extract, Group E were control . Extract concentrations of 50 mg, 100 mg and 150 mg were given to three subgroups of each treatment group for 30 days. Birds were then challenged with intramuscular administration of 0.2 ml of 10^6 ELD_{50} (50 percent Embryo Lethal Dose) of saline suspension of NDV on the 30th day, and were examined for clinical signs and symptoms (though not reported in this article), micro-flora of the trachea were specifically noted.

2.10 Bacteria isolation

Trachea swabs were sampled from the nineteen (19) groups including the control groups. They were transported to Microbiology and Parasitology Laboratory Unit, Department of Medical Laboratory Science, Achievers University, Owo. All solid and liquid media used in the laboratory were products of Biotec Limited (code MM 1008s). Swabs were initially plated into two 5% blood agar plates, one CLED (Cystine Lactose Electrolyte Deficient) agar plate, one *Staphylococcus/Streptococcus* selective agar plate and a section of Sabouraud agar (Chessbrough 2007). One of the blood agar plates was then incubated under anaerobic conditions (CO_2 jar) and the other four plates were incubated in aerobic condition at 37 ^{0}C for a maximum of 24h. The plates were then examined and colony types were recorded. Further basic identification tests including Gram stain, oxidase and coagulase, and battery of sugars reactions were carried out to confirm genus and species. Sensitivity testing was performed using Bauer-Kirby technique (Baker and Breach, 1980) and zones of inhibition in diameters (mm) around the antibiotic impregnated discs were measured and organisms designated sensitive or resistant according to laboratory protocol.

2.11. Statistical Analysis

Results presented are means ± SEM of eight independent determinations. Results obtained from this study were statistically analyzed using one way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) using SPSS 17.0. Significant differences between the treatment means were determined at 95% confidence level.

3.0 Results

3.1 Bacterial Isolate

The Prevalence of bacteria isolates among broiler chickens is shown in Table 1. A total of 65 (68.4%) yielded mixed growth of bacteria in most samples cultured and axemic culture was employed to grow pure culture before isolate identification and drug testing, and only one sample yielded growth of yeast cell. The frequency of bacteria isolation are, *Pseudomonas aeruginosa* (23.1%), *Enterococcus feacalis* (23.1%) and *Staphylococcus spp* (23.1%), then coliform bacteria (30.8%) included lactose-fermenters species: (LF) *E. coli* and Non lactose-fermenters (NLF) *Klebsiella* species. The most common isolates from different groups of treatments were coliform (both LF and NLF), followed by *Enterococcus*, *Staphylococcus* species and *Pseudomonas* species that recorded same frequency of occurrence. Only 9 (13.9%) of the total population isolated resisted the test antibiotics among which are *Enterococci spp.* (2), *Pseudomonas aeruginosa* (2) and coliform (5) showed resistance to either cerofloxacin or genticin with disc diffusion methodology used in this study. Yeast (*Candida albicans*) growth was observed in one of the negative control group. The frequency of bacteria isolation from broilers challenged with NDV following supplementation and control group are shown in Figure 1. *Aloe vera* recorded 11 (17.0%), *Alma millsoni* 15 (23.0%), *A. marginata*, *G. lucidum* and control group recorded 13 (20.0%) each. There was no statistically significant difference ($P<0.05$) in the occurrences of bacteria in trachea of chickens supplemented with different concentration of the extracts, both challenged with NDV and unchallenged groups.

4.0 Discussion

It is time to recognize the true cost of antibiotic use in animal practice in terms of antibiotic resistance and its consequences on the sustainability of susceptible bacterial flora in the environment and to act accordingly. Though, the majority of birds in our study were laden with ND following viral challenge of their immunity, which may have biased the results. The expected tracheal flora of healthy broilers chicken is not well-documented. The total distribution of Gram positive and Gram negative bacteria was ratio 6 : 7, there were differences in distribution of bacteria in the broilers and the result reveal that Gram negative bacteria were more than half of the total isolates. Whereas only 46.2 % of the bacteria isolated from broiler were Gram positive organisms. *Coliforms*, *Enterococcus*,

Staphylococcus aureus and *Pseudomonas* spp. were found to be predominant in trachea of broiler chicken used in this study, and these organisms may not be pathogenic to the birds as there were no signs of bacterial infection on the trachea. Despite the supplementation at different concentrations administered both ND challenged and control groups yielded growth of coliform (LF). *Enterococcus* species are generally considered to be of low pathogenicity and can form part of the normal flora of some mucosal surfaces. It is known to exhibit resistance to common antibiotics and if found sensitivity to drug testing is considered to be clinically important. This finding is in agreement with the report of Selbitz (1992) and Monks *et al*. (2005). The finding on viral laden broiler chickens is also in agreement with the report of Reavill (2003); he found Coliforms, *Enterococcus, Pseudomonas* and *Staphylococcus* species as common tracheal isolates, importantly, though these bacteria were also isolated from the control groups that did not show evidence of nasal congestion.

The diameter of zone of inhibition may indicate that the isolates possess no threat to the birds, since the duo antibiotics tested are the commonly used antibiotics by farmers to control bacterial infection in the farms and reduced mortality rate of bacteria origin within the birds. Also the continued use of antibiotic may encourage micro- flora substitution and replacement with more virulent types. Therefore, care must be taking in the treatment of bacterial infection among poultry birds as non-pathogenic organism in the trachea may acquire resistance from a transposons and other mobile elements, plasmid, it may be mediated chromosomal mutation. Mims *et al*. (1999) further explained that multiple resistance genes can come together in a structure called integron instead of existing separately on chromosome, plasmid or transposon. The integron encodes a site-specific recombination enzyme, which allows insertion as well as excision of antibiotic resistance gene cassettes in the integron attachment site. An integron may contain up to eight resistance genes (Rowe-Magnus and Mazel, 2002). Thereby resulting to threat from the pathogenic strain and resist the commonly used drugs. It may also predispose the birds to upper respiratory tract infection. The problems of drug resistance isolate potent danger to the farmers and the entire community in case of outbreak of poultry related disease.

The isolation of yeast cell from negative control group may be a sign of immune insolvency or impaired immunity of that group of birds as they were neither vaccinated nor supplemented with extracts. However, there is therefore the need to further study poultry gut flora, detail could reveal their roles and/or pathogenicity to birds, significance to human (handlers) and public health risk associated with domestic birds keeping and poultry sited in close proximity to human dwellings.

Conclusion

Results showed array of microbial flora in the trachea of broiler chickens, the expected tracheal flora of healthy broiler chickens are not well-documented. *Coliforms, Enterococcus, Staphylococcus aureus* and *Pseudomonas* spp. were found to be general in trachea of broiler chickens used in this study and these organisms may not be pathogenic to the birds as there were no other signs of bacterial infection on the trachea. Though, isolates were sensitive to genticin and cerofloxacin, there may be yet, public health risk associated with domestic birds keeping and poultry sited in close proximity to human dwellings.

Conflict of Interests

The authors do not have a direct financial relationship with the commercial identity mentioned in this paper.

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Table 1. The Prevalence of bacteria isolates among broiler chickens

Isolates	Frequency of isolation (%)	No of isolates sensitive to antibiotics (%)	No of isolates resistant to antibiotics (%)	Zone of inhibition in diameter (mm^3)	
				Genticin	Cerofloxacin
Pseudomonas aeruginosa	15 (23.1)	13 (86.7)	2 (13.3)	17 ±0.5	17 ±0.5
Staphylococcus aureus	15 (23.1)	15 (100.0)	0 (0.0)	15 ±0.5	16 ± 0.5
Enterococcus feacalis	15 (23.1)	13 (86.7)	2 (13.3)	19 ±0.5	17 ± 0.5
coliform (LF): ***E. coli*** and (NLF): ***Klebsiella spp.***	20 (30.8)	15 (75.0)	5 (25.0)	20 ±0.5	21 ± 0.5
Total	65 (100.0)	56 (68.4)	9 (13.9)		
Controls:					
1)***S. aureus*** (NCTC 6571)	-	1	-	21 ±0.5	21 ± 0.5
(2) ***E. coli*** (NCTC 10418)	-	1	-	22 ±0.5	21 ± 0.5

Key: **NCTC** = National Collection of Type Cultures
LF= lactose fermenter, NLF = non lactose fermenter (statistical significant $P < 0.05$)

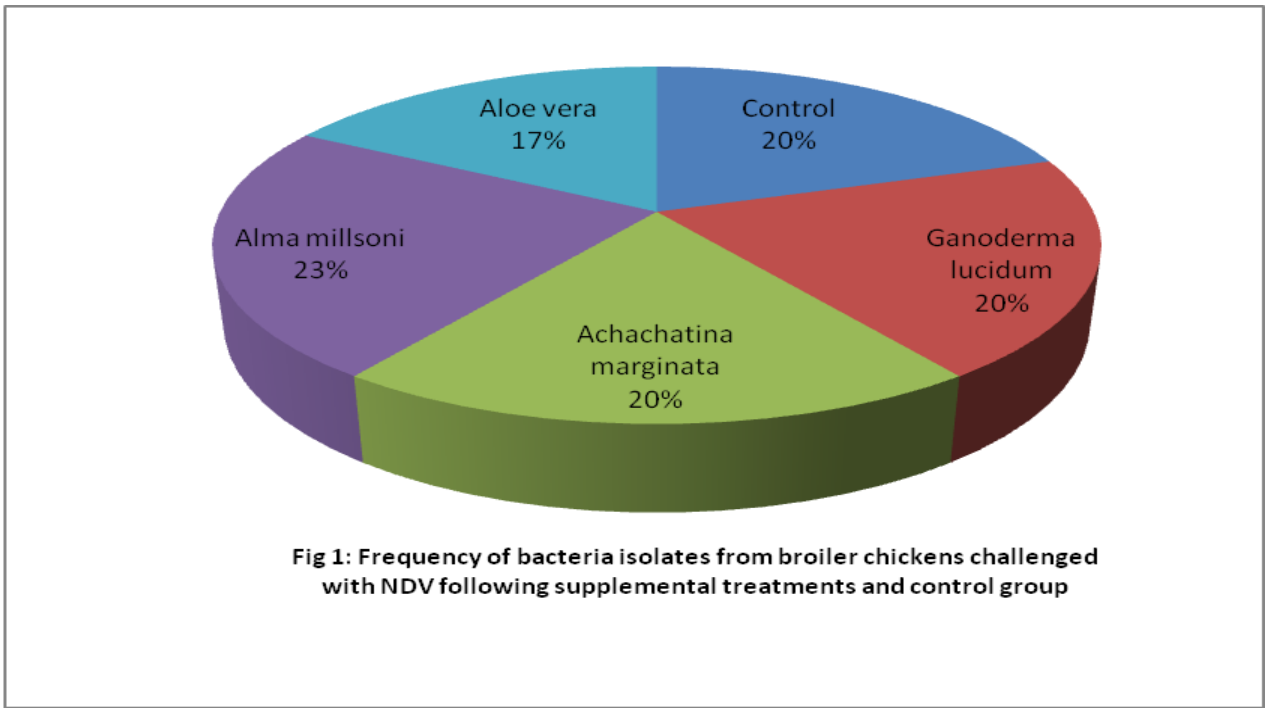

Fig 1: Frequency of bacteria isolates from broiler chickens challenged with NDV following supplemental treatments and control group