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Molecular Based Survey of Pathogens Associated with Respiratory Disease Outbreaks in Broiler Chickens in Accra

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Abstract
Respiratory diseases in poultry are caused by a host of pathogens that act either singly or in association with each other. The clinical signs elicited by some poultry respiratory pathogens are similar and may confuse diagnostic efforts. Definitive diagnoses involving the use of sensitive, robust and rapid laboratory method are important for the identification and differentiation of specific agent(s) during infection. Our study aimed at identifying the involvement of Infectious Bronchitis Virus, Newcastle Disease Virus and *Mycoplasma gallisepticum* (MG) in outbreaks of respiratory diseases in chickens in the Ga-East district of Greater Accra using the Polymerase Chain Reaction technique (PCR). Twenty (20) trachea swabs were obtained from four chicken flocks (5 per flock) with acute respiratory diseases. RNA and DNA were extracted from each sample individually. The S1 glycoprotein gene, MgC_2 gene and fusion protein gene of IBV, MG and NDV respectively, was individually target in a single tube PCR reaction. Amplicons were resolved on a 1% agarose gel stained with Ethidium bromide and visualized with a UV trans-illuminator. Twenty percent (20%) of samples tested negative to all three pathogens. IBV and NDV as single agents were detected in 40% and 20% of the samples respectively. Twenty percent (20%) of the samples were found to be co-infected with IBV and NDV. None of the samples tested positive to MG. IBV and NDV play major roles in outbreaks of respiratory diseases in commercial broiler chickens in Ga-East district of Accra.
Keywords: Respiratory pathogens, Broilers, Ghana, Polymerase Chain Reaction

1. Introduction

Respiratory tract infections are of paramount importance in the poultry industry. This is because large numbers of birds may be lost in poorly managed cases with subsequent significant economic losses to the industry. In addition to mortality, morbidity is usually high. Respiratory tract infections increase the overall cost of production in terms of the provision of services of qualified veterinary personnel and the cost of medication for possible treatment. It is therefore important to reduce if not eliminate, respiratory infections among poultry flocks to the barest minimum to have good production and maximize profit of the producer. Poultry respiratory diseases are known to be caused by a host of pathogens including Newcastle disease virus (NDV), *Mycoplasma gallisepticum* (MG) and Infectious Bronchitis Virus (IBV) with associated significant economic losses to the industry. These respiratory pathogens are of major importance because they can cause disease independently, in alliance with each other or in association with other bacterial and viral agents (Ali & Reynolds 2000; Yashphal *et al.* 2004). Appropriate vaccination coupled with good Management practices such as provision of good Biosecurity, are practical ways of controlling these pathogens in a flock. Vaccination reduces the level of clinical diseases and promotes optimal performance of the poultry birds.

Newcastle Disease remains endemic in Ghana with annual mortality of indigenous chickens remaining between 80-90% (Awuni J.A. 2002; Aboe *et al*. 2006). The incidence of Newcastle disease in commercial chickens is however low. This could be because commercial chickens are regularly vaccinated against the disease. Vaccination against Infectious Bronchitis Virus and *Mycoplasma gallisepticum* are not carried out. This is probably because of the limited information on IBV and MG in the poultry industry in the country and the absence of appropriate control interventions. In a recent study on MG, Ayim *et al*. (2012) reported of high prevalence (59.1%) of *Mycoplasma gallisepticum* infection in layer chickens in Accra. The study also reported of a significant increase in prevalence with an increase in age of the layer flock. However the work did not confirm the involvement of MG in respiratory disease outbreaks in chickens in the area. Using the Reverse Transcriptase Polymerase chain reaction technique, Ayim-Akonor *et al*. (2013) identified Infectious Bronchitis Virus as a major pathogen involved in the frequent outbreaks of respiratory diseases in chickens in Accra. This work provided an initial evidence of the presence of IBV in the country and confirmed its involvement in respiratory diseases in chickens in the study area. Studies have shown that the incidence and severity of diseases are dependent on among others, the presence of other pathogens and their interactions with each other (Dennis & Dennis 2008). It is therefore important to identify the actual pathogen(s) involved in outbreaks of diseases particularly in situations where similar clinical symptoms are elicited by these pathogens. These would help in the development and implementation of appropriate interventions to mitigate the effect of these pathogens with

subsequent positive impact on productivity.
The aim of this study was to establish the single or joint role(s) of NDV, IBV and MG in outbreaks of respiratory diseases in broiler chickens in Accra.

2. Materials and Method

2.1 Study site and flock description
The study was undertaken in the Ga East District, an urban community in the Greater Accra region of Ghana from November, 2011 to January, 2012. Small-scale commercial poultry production is intensively carried out in this district. Farmers mostly keep layers interspersing it with broiler production during certain festive seasons. During the three (3) month period, the team examined six (6) commercial broiler chicken flocks suffering from acute respiratory disease. The broiler chicken vaccination programme in Ghana includes 2 live vaccines of NDV at week 2 and week 6 administered via drinking water and 2 Infectious Bursa Disease Virus administered at 1st and 3rd week also through drinking water. None of these flocks was vaccinated against *Mycoplasma gallisepticum* and *Infectious Bronchitis*.
2.2 Sampling
From each diseased flock on a farm, five (5) birds were randomly handpicked for sampling. A cotton swab was used to firmly swab the trachea of the birds using one (1) cotton swab per bird. Swab was immediately put in a labeled 2ml Eppendorf micro centrifuge tube containing 500µl of 0.1M PBS and firmly closed. Samples were put in a cool box and transported to the Molecular Biology Laboratory of the Animal Research Institute (ARI) for further processing.
2.3 Viral RNA extraction
The tube containing the swab was vigorously vortexed for 20sec.Sixty microlitres (60µl) of this sample was pipetted using a 200µl barrier filtered pipette tip into a pre-labeled 2ml sterile micro centrifuge tube. The Qiagen RNeasy Mini Kit was used to extract RNA from all the samples following the manufacturer's instructions. RNA was eluted in 60µl RNase free water. The extracted RNA was stored at -20°C until needed. The Massachusetts antigen (M-41) (GD, Health Services, Deventer, Netherlands), and La-Sota (Intervet International, Holland) were added as IBV and NDV positive extraction controls respectively. PBS (1X) was added as a negative extraction control.
2.4 DNA extraction
From each swabbed tube, 200 µl of sample was collected into a sterile 2ml micro tube. DNA was extracted using the Qiagen DNA extraction kit, following the manufacturer's instruction. MG vaccine 6/85 and PBS (1X) were used as positive and negative extraction controls respectively.
2.5 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for IBV and NDV detection:
Each RNA extract was tested in two separate tubes for IBV and NDV. IBV forward primer LC3 5′-ACA GAT TGC TTG CAA CCA C-3' and reverse primer LC5 5′-ACT GGC AAT TTT TTC AGA-3' were used. For NDV detection, forward primer NDVU 5'-GGA GGA TGT TGG CAG CAT T-3' and reverser primer NDVD 5'-GTC AAC ATA TAC ACC TCA TC-3' were used.
The Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for both NDV and IBV was individually performed in a 200µl thin walled PCR tube in a final reaction volume of 25µl. The One-Step RT PCR kit from Qiagen was used following the manufacturer's instructions with some slight modifications. Briefly into each separate tube, 13µl of RNase free water was added. This was followed by the addition of the following: 5µl of 5X One-Step RT-PCR buffer, 1µl dNTP mix (10mM), 1.25µl appropriate forward and reverse primer (10pmol of each primer) and 1 µl enzyme mix (5U/µl). 2.5µl RNA extract was added. The amplification was carried out in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems). For cycling conditions: Reverse Transcription for synthesis of complimentary DNA was performed at 45°C for 60min, followed by 94°C for 5 minutes to inactivate Reverse Transcriptase enzyme and activate the Taq polymerase. This was followed by 3 step amplification of 40 cycles of denaturation at 94°C for 45s, annealing at 51°C for 1 min (for IBV) and 58°C for 1min (for NDV) ,extension at 72°C for 90s and a final extension at 72°C for 5mins. Four controls were added in each reaction set. These were extraction negative control (1X PBS), extraction positive control (M-41, for IBV and La-Sota for NDV), PCR positive control (M-41, for IBV and La-Sota for NDV) and PCR negative control (nuclease free water).
2.5 PCR for *Mycoplasma gallisepticum* detection
The forward and reverse primer pair, MgC_2F: 5′-CGCAATTTGGTCCTAATCCCCAACA-3′ and MgC_2R: 5′-TAAACCCACCTCCAGCTTTATTTCC-3′ respectively, targeting the MgC_2 gene of *Mycoplasma gallisepticum* were used. Reaction was performed in a 200µl thin walled PCR tube in a final reaction volume of 25µl. The Qiagen PCR kit was used. Into each tube, the following was added: 14.25µl of RNase free water, 2.5µl of 10X buffer, 1.5 µl $MgCl_2$ (25Mm), 0.5µl dNTP mix (10mM), 1µl MgC_2 forward primer (10pmol), 1µl MgC_2 reverse primer (10pmol) and 0.25µl Taq polymerase (5U/µl). 4µl DNA extract was finally added. The amplification was

carried out in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) with the following cycling conditions: one cycle at 94°C for 5 mins, 3 step amplification of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 2 mins for 40 cycles and a final extension at 72°C for 5 mins.
Four controls were added in each reaction set. An extraction negative control (1X PBS), extraction positive control (MG antigen, NVSL, USA), PCR positive control (MG antigen, NVSL, USA) and PCR negative control (nuclease free water).
2.6 Agarose Gel Electrophoresis and Visualization of DNA amplicons:
A 1% agarose gel was prepared in a conical flask by weighing, 0.6g of agarose powder (Invitrogen) and measuring 60mls of 0.5X TBE buffer. This was dissolved in a microwave oven for a total of 4 minutes, pausing to swirl the flask intermittently. 5µl of ethidium bromide (5ug/ml) was added to the melted agarose and swirled to mix well. The mixture was poured into the gel tray fitted with gel combs. Agarose was allowed to solidify after which combs and blocks were removed. 0.5X TBE buffer was added to the gel tank to cover the solidified agarose gel. 5 µl of 6X gel loading dye were added to the PCR product in each tube to make a final concentration of 1X. 5µl of 100bp ladder (1µg/ml) (Invitrogen) was loaded into the first well while the remaining wells were loaded (1 sample per well) with 10µl of the sample containing the dye. Electrophoresis was carried out at 60V for 60 min using Biorad sub-Cell GT system. DNA amplicons were visualized on a High Performance UV trans-illuminator manufactured by UVP and photographed.

3.0 Data Analysis
Data was entered in Microsoft excel, 2007 and analyzed using descriptive statistics. Frequency distribution was used to compare pathogens detected and the trends highlighted graphically.

4.0 **Results and Discussion**
4.1 Results
Characteristically, affected flocks were depressed, weak and reluctant to move. Feed intake was observed to be poor. Tracheal rales, cough, sneeze and air gasping were commonly seen among the flocks. In two (2) farms, greenish to brown watery diarrhoea was seen at the farm and whitish paste at the vent of some birds.
The age of the birds showing acute respiratory disease was at a minimum of 5 weeks and a maximum of eight (8) weeks. The primer pair LC3 and LC5 amplifies the S1 glycoprotein gene of IBV given a product size of 383bp (Figure 1). The fusion protein of Newcastle disease virus was amplified using the primer pair NDVU and NDVD. This yields a 320bp PCR product (figure 2). The MgC_2 gene of MG was detected with primer pair MgC_2 F and MgC_2 R. This gives a product size of 273bp (not shown).

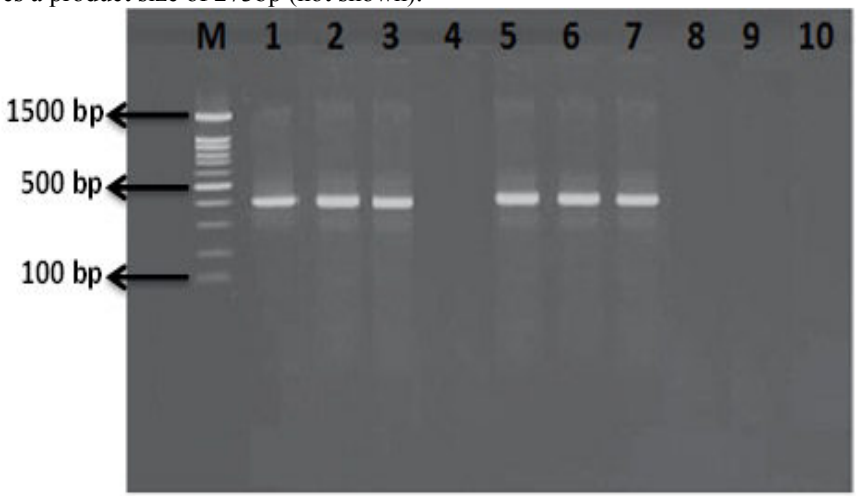

Figure 1: 1% agarose gel stained with Ethidium bromide. Lanes M: 100bp ladder, 1: positive extraction control (M-41); 2: positive PCR control (M-41); 9: negative extraction control (PBS); 10: negative PCR control (nuclease free water); 3, 5, 6, 7: positive samples; 4, 8: negative samples

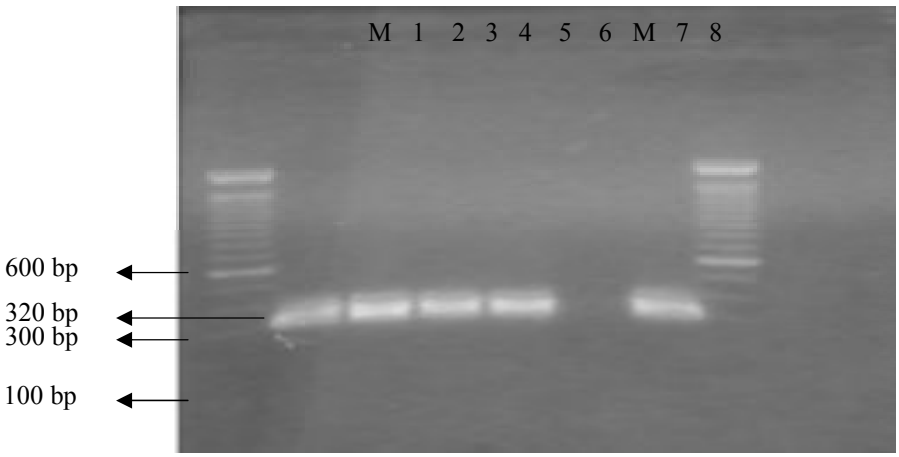

Figure 2: 1% agarose gel stained with Ethidium bromide. Lanes M: 100bp ladder, 1: positive extraction control (La-sota); 2: positive PCR control (La-sota); 7: negative extraction control (PBS); 8: negative PCR control (nuclease free water); 3, 4 and 6, positive samples; 5 negative sample

A total of twenty (20) tracheal swabs were analysed from broilers with acute respiratory disease for NDV, IBV and MG. The MgC_2 gene was not detected in any of the twenty (20) samples tested. The fusion and S1 glycoprotein genes were in total detected in eighty percent (80%) of the test samples. Infectious Bronchitis Virus, as a single agent was detected in forty percent (40 %) of the test samples whiles Newcastle Disease Virus also as a single agent was detected in twenty percent (20%) of the samples. Twenty percent (20%) of the samples were detected to be co-infected with Newcastle disease virus and Infectious Bronchitis Virus. Four samples (20%) tested negative to MG, IBV and NDV (Figure 3).

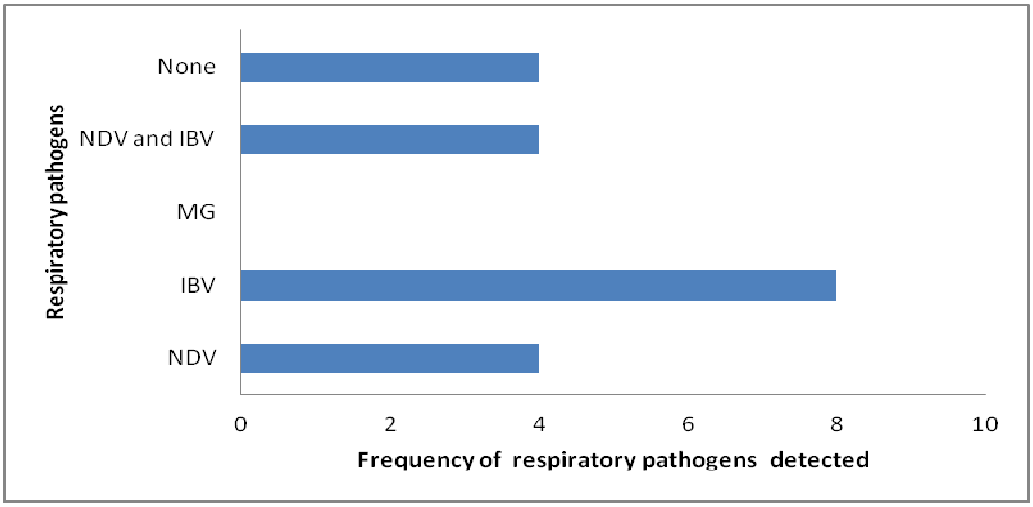

Figure 3:Distribution of respiratory pathogens detected in broiler flock

NVD: Newcastle Disease Virus, IBV: Infectious Bronchitis Virus, MG: *Mycoplasma gallisepticum*

4.2 Discussion

Respiratory diseases in poultry are known to be caused by several microorganisms that either act singly or jointly with several other agents to cause diseases (Sakuma *et al*. 1981; Yashpal *et al*. 2004). The clinical signs elicited by some poultry respiratory pathogens are similar and may confuse diagnostic efforts. Definitive diagnoses involving the use of sensitive, robust and rapid laboratory method are important for the identification and differentiation of specific agent or agents during infection. This enhances diagnostic efforts prompting the implementation of appropriate strategies.

Rapid advances in Molecular biology techniques have permitted the development of specific and sensitive tests for rapid diagnosis of diseases and differentiation of closely related organisms. The application of the PCR technique in disease diagnosis is a rapid and robust methodology. It however requires specialized equipments and well trained personnel both of which are often limited in many developing countries. These limit the application of this technique especially in the area of animal health.

The primers used for the amplification reactions of the three respiratory pathogens have previously been published by Stauber *et al.* (1995) for NDV, Jackwood *et al*. (2003) for IBV and Hantow *et al.* (1998) for MG.

Samples were received from flocks that were 32 days and above and suffered from acute respiratory problems, suggesting that, these flocks have been exposed to pathogens in the field.

Infectious Bronchitis Virus (IBV) is an RNA virus belonging to the family of Coronaviridae. The viral genome is enveloped, positive sense and single stranded with a genome size of about 27kb (Cavanagh & Naqi, 2003). IBV causes respiratory infections in chickens with lesions in the trachea. It is worldwide in distribution in commercially producing chicken areas. However, as an RNA virus IBV has a high tendency to mutate. This frequent mutation has resulted in the recognition of over 20 serotypes worldwide with minimal to no cross protection (Jack & Jackwood 2008). Cook (2008) proposed a careful and correct administration of live attenuated and inactivated vaccines as an effective control strategy of infection among flocks. This is because vaccines stimulate the production of antibodies in the upper respiratory tract of the birds and protect them against IBV and reduce viral shedding.

Although none of the birds had been vaccinated against Infectious Bronchitis Virus, IBV was the most frequently detected pathogen. IBV was detected in a total of twelve (60%) of the test samples (Figure 3). Of these, eight (66.7 %) were found to be single infection whereas in four (20%) samples, IBV was observed to be combined with Newcastle disease virus. The high percentage of IBV detected, indicates the endemic nature of the pathogen in the study area. This could be attributed to the high susceptibility of broiler flocks to IBV (Cavanagh & Gelb 2008). The high occurrence of IBV could also be attributed to the absence of control interventions against IBV in the area as a result of the limited knowledge of the pathogen and extent of its activity in the country. High prevalence of IBV in chicken flocks have been reported in some African countries, an indication of the endemic nature of the pathogen in poultry producing areas on the continent and the need for implementation of control strategies (el Houadfi & Jones 1985; Abdel-Moneim *et al.* 2006; Mushi *et al.* 2006 ; Emikpe *et al.* 2010).

Newcastle disease virus; a Paramyxovirus has a negative sense, single stranded, nonsegmented RNA genome. Clinical signs of NDV vary widely depending on factors such as viral strain, host species, age of the host, and immune status of host and the presence of other pathogen. Some strains cause sudden death with 100% mortality and others cause subclinical infections. Respiratory infections are a major clinical sign of NDV (Dennis & Dennis, 2008). Newcastle Disease Virus was the second highest pathogen identified to be the cause of respiratory disease in the affected chickens. In total NDV was detected in forty percent (40%) of the analyzed samples, of which fifty percent was a single infection and the other fifty percent (50%), a co-infection with IBV (Figure 3). The second NDV vaccination for broilers is administered in the 6th week. The La-Sota vaccine given is a live attenuated vaccine. The detection and involvement of NDV in respiratory disease outbreaks in the vaccinated flocks could indicate that probably, the broilers were naturally exposed to a more virulent field strain of NDV that the vaccine strain could not protect the birds against or secondly, that there was a failure in the vaccination of these birds against Newcastle disease. The Newcastle Disease Virus identified in the present study was not characterized to establish the actual source of the pathogen, that is, either a vaccine strain or a wild type. Again, antibody titre levels before and after vaccinations were not determined to ascertain the success or otherwise of the vaccine administered. These were not the focus of the study. However, similar observations of outbreaks of respiratory diseases among vaccinated flocks have been reported in many poultry producing areas (Ebrahimi *et al*. 2001; Arfan *et al*. 2008; Roussan *et al*. 2008).

Mycoplasma gallisepticum (MG) is the most pathogenic and economically significant mycoplasmal pathogen of poultry causing chronic respiratory disease in chickens and sinusitis in turkeys. Infection with MG increases carcass condemnation at slaughter reduces feed and egg production and reduces hatchability of fertile eggs (Ley 2008). MG is a prokaryote devoid of a cell wall and a member of the class Mollicutes (Razin 1992; Razin *et al.* 1998). None of the samples tested positive to *Mycoplasma gallisepticum* implying that MG was not involved in the respiratory disease outbreaks seen in the broilers during the period of study. Although Ayim and colleagues. (2012) reported of high levels of antibodies in the sera of layer flocks in the study area, an indication of a high activity of the pathogen, MG was not implicated in the current respiratory condition seen in the broilers in the area. The presence of antibodies in an apparent healthy animal does not necessarily indicate a present activity of the pathogen and thus could account for the non detection and hence noninvolvement of MG in the current respiratory outbreak (Morrow 2008).

Twenty percent of the samples were diagnosed as negative for all three pathogens, thus excluding any of these pathogens as the cause (s) of the respiratory disease seen in the chickens. Other known poultry respiratory pathogens such as *E. coli*, Avian Influenza, and Avian pneumovirus could be responsible for the respiratory diseases in these chickens. The detection of all the respiratory pathogen(s) possibly involved in the respiratory disease outbreaks was not the scope of the study.

5. Conclusion

Our study has shown that Infectious Bronchitis Virus and Newcastle disease virus are significant cause of respiratory diseases outbreaks in broiler chickens in the Ga -East district of Accra. Both pathogens act singly and in association with each other to cause the disease in chickens in the district. Molecular techniques are a valuable laboratory tool that can aid in rapid diagnosis and identification of actual pathogen(s) during infections for prompt treatment. IBV needs to be isolated and characterized and appropriate intervention instituted to reduce the effect of this pathogen. Although NDV vaccination is regularly done, NDV associated with respiratory disease outbreaks should be characterized to establish the actual source of the pathogen (vaccine or wild type). This would help in the development of appropriate intervention strategy.

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