SAARC J. Agri., 10(2): 95-105 (2012)

CYTOLOGICAL EVALUATION OF NECROPSY GUIDED IMPRESSION SMEARS OF CHRONIC RESPIRATORY DISEASE OF CHICKENS

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ABSTRACT

The study was conducted to develop a cytology based diagnostic tool for the diagnosis of avian *Mycoplasma* and *E. coli* infections at post mortem in the field condition. A total of 38 culture and PCR confirmed *Mycoplasma*, *E. coli* or mixed infected samples were used for this study. Lung impression smears were prepared on glass slide from the samples at post mortem examination. Inflammatory cells were counted on microscope after Giemsa staining. Cell counts were analyzed with Bonferroni joint confidence interval and Mann-Whitney U test. The average cell percentages in healthy cases were 73.54-81.66%, 9.63-13.37% and 7.42-14.38% for lymphocyte, heterophil, and macrophage, respectively. In case of *Mycoplasma* infection, average percentages of lymphocyte, heterophil and macrophages were 82.01-88.10%, 5.6 to 8.16% and 4.52 – 9.68%, respectively. In *E. coli* infection, average percentage of lymphocyte, heterophil and macrophages were found as 64.44-70.76%, 19.73-23.47% and 8.9-12.7%, respectively. In mixed infection, lymphocyte, heterophil and macrophage were found as 76.08-80.50%, 13.47 –17.63% and 4.56 –7.66%, respectively. Statistical analyses revealed that in *Mycoplasma* infection number of lymphocyte and in *E. coli* infection number of heterophil increased significantly ($p < 0.01$). In MC complex, number of heterophil increased and macrophages decreased significantly ($p < 0.01$). These findings could help identification of *Mycoplasma*, *E. coli* or *Mycoplasma- E. coli* complex at post mortem examination in the field condition.

Key words: CRD, mycoplasma, *E. coli* and cytology.

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Received: 07.05.2012

INTRODUCTION

Poultry rearing is playing a vital role in the poverty alleviation and income generation in Bangladesh. Mycoplasmosis is an economically important disease of poultry caused by four commonly recognized pathogens – *Mycoplasma gallisepticum*, *M. synoviae, M. meleagridis and M. iowae*. *M. gallisepticum* and *M. synoviae* (Sikder *et al.,* 2005) causing chronic respiratory disease (CRD) with significant mortality and decrease in egg production in chickens, turkeys and other avian species. In Bangladesh, the prevalence of Mycoplasmosis markedly increases in the winter season and may reach up to 61.45% which is a threat for poultry sector (Sikder *et al.,* 2005). Very often it is confused with other respiratory diseases particularly with *E. coli* infection which sometimes make the treatment very expensive. Early diagnosis is the corner stone for the successful treatment, prevention and control strategies. The organism can be diagnosed by isolation, serological tests, culture and nucleic acid based tests (Nascimento *et al.,* 2005). Conventional means of isolation or culture have proven to be tedious and time consuming and it requires special growth supplement, which make it an expensive option (OIE, 2008). It is important to choose a way for diagnosis that will be compatible with actual field condition, as well as facilities available. Clinical pathology has played a major role in the diagnosis of human and animal diseases. The importance and accuracy of cytological examination as a diagnostic tool has been studied and reviewed in the human and veterinary literature (Bottles *et al.,* 1986, Cochand-Priollet *et al.,* 1987, Campbell, 1988, Cowell *et al.,* 1989) as an indirect method of diagnosis in case of infectious diseases which involve relatively non-invasive means of sampling from the specimens and could provide one step forward from post mortem diagnosis. This paper describes the use of cytological smear collected at necropsy for the diagnosis of avian *Mycoplasma* and *E. coli* infections which were compared with the histopathological changes.

MATERIALS AND METHODS

Samples

A total of 38 samples (Blood, lung exudates, swab from trachea and larynx) were collected from dead birds that were suspected to be the case of *Mycoplasma* or *E. coli* infections during November, 2008 to June, 2009. Ten (10) samples were collected from apparently healthy chickens at regular slaughter that assumed to be free from chronic respiratory diseases clinically as control. The samples that were confirmed as *Mycoplasma or E. coli* or mixed infection by culture and PCR were subjected for cytological evaluation.

Pathological Studies

Gross pathological examination was conducted on necropsy. Tissues from lungs, liver, trachea, heart and spleen organs were collected at necropsy and were preserved as per requirement. Part of the samples was fixed in 10% neutral buffered formalin for histopathological examination as per earlier described procedure (Luna 1968).

Cytology

Clean glass slides were gently touched onto the lesion of lung and immediately lifted off and air dried, stained with Giemsa staining and observed under a microscope using a 100× magnification with oil immersion. The counting was done gradually from one side to another side and numbers of inflammatory cells were estimated by counting 300 cells. Only *Mycoplasma, E. coli* or complicated *Mycoplasma* infected cases determined on necropsy findings were considered under this count.

Isolation of *Mycoplasma* and *E. coli*

Lung and tracheal swabs were collected either in nutrient or Mycoplasma broth supplemented with supplement G (Oxoid, Milan, Italy) and kenamycin. The nutrient broth containing suspected swab was incubated in aerobic condition at 37°C for 48 h. Upon growth of the organism, 100 µl of inoculums (infected media) were inoculated into EMB agar plate (Oxoid, Milan, Italy). Plates were incubated at 37°C for 48 h. After 48h, production of typical metallic sheen in EMB agar was considered as *E. coli* infection. Mycoplasma broth (Oxoid, Milan, Italy) containing suspected swab was incubated at 37°C for 72 h. After growth, 100 µl of Inoculum (infected media) were inoculated into Mycoplasma agar (Oxoid, Milan, Italy) with 20% of equine serum and plates were incubated for 10 days at 37°C in humid chamber with 10% CO_2. After 2 or 3 passages *Mycoplasma*, when present, produced typical colony with fried egg appearance (Figure.1).

Identification of isolated *Mycoplasma* using PCR

Polymerase chain reaction (PCR) was used to detect the organisms following the procedure described by Lauerman *et al.,* (1995). Commercial PCR kit **(**PCR Master Mixture Kit, GeNei™, Banglore Genei, BDA Industrial Suburb, Peenya, India) was used for this purpose. *Mycoplasma gallisepticum* vaccine; MG TS-11(Merial Select, Inc, Gainesville, USA) was used as positive control for *Mycoplasma gallisepticum* during PCR. Wizard R Genomic DNA Purification Kit (Promega Corporation. 2800 Woods Hollow Road. Madison, USA) was used to extract the DNA from *Mycoplasma* vaccine and *Mycoplasma* isolates as per manufacturer's instructions. Primers and thermal profile were adopted from previous work (Lauerman *et al.,* 1995). DNA amplification was performed in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf, Germany). The amplified products were analyzed by 1.5% agarose gel (Promega Corp. Madison, WI, USA). The gel was visualized under UV light on a transilluminator (Labortechnik, Germany). The expected product size of *M. gallisepticum* and *M. synoviae* were of 900 and 497bp, respectively.

The diagnoses of the samples were made based on the following criteria (Table 1):

Table 1: Criteria to identify *Mycoplasma* and *E. coli* infections

Diagnosis	Cultural characteristics/ PCR detection
Mycoplasma infection	Samples showed typical *Mycoplasma* colonies in *Mycoplasma* agar and PCR positive
E. coli infection	Samples showed positive growth with metallic sheen in EMB agar and no colonies in *Mycoplasma* agar.
Mycoplasma and *E. coli* complex	Samples showed positive growth with metallic sheen in EMB agar and typical *Mycoplasma* colonies in *Mycoplasma* agar and PCR positive.

Statistical Analysis

Cell counting results obtained from blood of *Mycoplasma* and *E. coli* infected birds and healthy control birds were analysed with Bonferroni joint confidence interval, Mann-Whitney U test (Johnson and Wichern, 2002) using the software MS Excel, SPSS and MINITAB 13.

RESULTS AND DISCUSSION

The samples that were confirmed as *Mycoplasma or E. coli* or mixed infection either by culture (Figure.1) or by PCR (Figure.2) were subjected for cytological evaluation. Pathological study was done to correlate the lesions with cytological smears. For normal healthy cases the average lymphocyte, heterophil, and macrophage counts were 73.54-81.66%, 9.63-13.37% and 7.42-14.38%, respectively. Impression smears sampled from *Mycoplasma* infected birds showed predominant lymphocytic aggregates containing 82.01-88.10%, on an average 8% increase when compared with normal healthy cases. The average cell percentage of heterophil and macrophage was found as 5.60-8.16% and 4.52-9.68% respectively, average 5% decrease of both cells when compared to the normal healthy cases. On Mann-Whitney U test it has been found that increase of lymphocyte and decrease of heterophil count significantly differ at 5% or 1% level of significance in comparison to normal healthy birds. These cytological changes closely correlated with the histopathological lesions of the same *Mycoplasma* confirmed birds where pneumonia with predominant lymphocytic infiltration was observed (Figure. 3). On the other hand, impression smears from *E. coli* infected birds showed an increasing trend of heterophil count (19.73-23.47%), but lymphocyte and macrophage counts were relatively decreased (64.44-70.76% and 8.9-12.7%). These findings also correspond to the findings of a previous histopathological study (Chandrashekhar, 2008). In same study statistical analysis showed decrease of lymphocytes and increase of heterophils and these significantly differ at 5% or 1% level of significance in comparison to normal healthy birds in *E. coli* infections. In case of mixed infection (*Mycoplasma* –

E. coli complex), the average percent of lymphocyte was 76.08-80.58% in which the difference is insignificant in comparison to normal healthy cases. But, the percent of hetetrophils increased to 13.47-17.63% and the percent of macrophages relatively decreased to 4.56-7.66%. In *Mycoplasma –E. coli* complex, the increase of lymphocytes is insignificant whereas significant difference existed between normal and *Mycoplasma – E. coli* complex for increased neutrophils and decreased macrophages. The relative numbers of different inflammatory cell types and their average percentage for normal healthy cases, *Mycoplasma*, *E. coli* infection and mixed infection are shown in tables 2 – 6. Figure 4 shows predominant lymphocytic infiltration in a lung impression smear of *Mycoplasma* infected birds.

Predominance of mononuclear inflammatory cells (lymphocytes and macrophages) and a smaller number of heterophil indicates a chronic inflammatory process. Since Mycoplasmosis is a chronic respiratory disease, increased number of lymphocyte has been found in this study. Increased heterophil indicates acute inflammatory response. *E. coli* infection is also considered as an acute infectious disease. The relative increase in the range of lymphocyte and heterophil and relative decrease in the range of macrophage indicate mixed infection. The results are in agreement with a previous study (Villiers and Dunn, 1998). Evaluation of cellular responses can provide the veterinarian with a diagnostic aid in the development of a presumptive or definite diagnosis (Campbell *et al.,* 1988). The present results provide a basis for the diagnosis and predilection of these diseases at post mortem. Although it is not confirmatory test, comparing with other diagnostic methods but advantageous to use the smear cytology due to its easiness as well as low expense and possible application for other histological test which is not possible on formalin fixed and paraffin embedded tissues. However, the cytological evaluation of *Mycoplasma/E. coli* infected birds has not been reported. When cytological diagnosis suggests *Mycoplasma/E. coli*, a more specific complementary examination should be added to confirm infection, so that adequate treatment can be done. This would probably reduce the treatment cost in poultry farming.

CONCLUSION

Cytological smear showed increased lymphocyte count (≥ 85%) which can be considered as *Mycoplasma* infection and an increased heterophil count (± 19%) can be considered as *E. coli* infection. In case of *Mycoplasma –E. coli* complex, normal lymphocyte ± 78% count and ±14% heterophil count can be considered. Cytological examination requires only glass slide and Giemsa stain; it could be a useful, rapid and compatible tool in field condition. Although it is not a confirmatory test, it could help pathologist to make decision one step further after post mortem examination that could guide a further confirmatory laboratory test.

REFERENCES

Bottles, K., Miller, T. R., Cohen, M.B. and Ljung, B.M. 1986. Fine needle aspiration biopsy. Has its time come? *Am. J. Med.,* 81: 525-531.

Campbell, T.W. 1988. *Avian hematology and cytology*. 1st ed., Iowa State University Press, Ames.

Cochand-Priollet, B., Chagnon, S., Ferrand, J., Blery, M., Hoang, C. and Galian, A. 1987. Comparison of cytologic examination of smears and histologic examination of tissue cores obtained by fine needle aspiration biopsy of the liver. *Acta. Ctyol.,* 31:476-480.

Cowell, R.L. and Tyler, R.D. 1989. *Diagnostic cytology of the dog and cat*. 1st ed., American Veterinary Publications, California.

Chandrashekhar, C., Taryn, K., Gurpreet, A. and Baljit, S. 2008. Lung responses to secondary exotoxin challenge in rats exposed to pig barn air. *J. Occup. Med. Toxicol.,* 3: 24.

Johnson, R.A. and Wichern, D.W. 2002. *Applied Multivariate Statistical Analysis*, Pearson Education, Inc., 5th edition, pp. 232-234.

Lauerman, L.H., Chilina, A.R., Closser, J.A. and Johansen, D. 1995. Avian *Mycoplasma* identification using Polymerase Chain Reaction Amplicon and Restriction Fragment Length Polymorphism Analysis. *Avian Dis*., 39: 804-811.

Luna, L. G. 1968. *Manual of histologic staining methods of the Armed Forces Institute of Pathology*. New York: McGraw-Hill Publications.200 pp.

Kanji, K.G. 1993. 100 *statistical tests*, SAGE Publications, pp. 86.

Nascimento, E.R., Pereira, V.L.A., Nascimento, M.G.F. and Barreto, M.L. 2005. Avian mycoplasmosis update. *J. Vet. Med*., 7: 1-9.

OIE (Office International des Epizooties/ World organization for Animal Health). 2008. Avian Mycoplasmosis. In *Mannual of Diagnostic Tests and Vaccines for Terrestrial Animals*. Chapter 2.3.5, pp: 482-496.

Sikder, A.J., Islam, M.A., Rahman, M.M. and Rahman, M.B. 2005. Seroprevalence of *Salmonella* and *Mycoplasma gallisepticum* infection in the six model breeder poultry Farms at Patuakhali district in Bangladesh. Cited from *Asian Network for Scientific Information,* pp 1682-8356.

Villiers, E. and Dunn. J. 1998. General Principal of cytological interpretation. *Companion Animal Practice.* pp. 429-437.

Table 2: Inflammatory cell count in cytological smear from lungs obtained from normal healthy cases.

Sample No	Lymphocyte (%)	Heterophil (%)	Macrophage (%)
N1	85	9	6
N2	75	10	15
N3	82	11	7
N4	75	15	10
N5	73	12	15
N6	77	13	10
N7	78	13	9
N8	76	9	15
N9	72	13	15
N10	83	10	7
**Mean(±Margin of error)	77.6(±4.06)	11.5(±1.87)	10.9(±3.48)

Table 3: Inflammatory cell count in cytological smear from lungs obtained from *Mycoplasma* infected cases.

Sample No	Lymphocyte (%)	Heterophil (%)	Macrophage (%)
M1	86	7	7
M2	85	8	7
M3	85	7	8
M4	80	9	11
M5	82	8	10
M6	90	6	3
M7	88	6	6
M8	86	6	8
M9	84	5	11
**Mean(±Margin of error)	85.11(±2.99)	6.88(±1.28)	7.1(±2.58)

Table 4: Inflammatory cell count in cytological smear from lungs obtained from *E. coli* infected cases

Sample No	Lymphocyte (%)	Heterophil (%)	Macrophage (%)
E1	69	19	12
E2	69	20	11
E3	71	19	10
E4	61	24	15
E5	70	21	9
E6	70	22	8
E7	69	21	10
E8	68	22	10
E9	67	23	10
E10	62	25	13
**Mean(±Margin of error)	67.6(±3.16)	21.6(±1.87)	10.8(±1.90)

Table 5: Inflammatory cell count in cytological smear from lungs obtained from mixed infected cases.

Sample No	Lymphocyte (%)	Heterophil (%)	Macrophage (%)
M+E1	75	17	8
M+E2	75	20	5
M+E3	78	15	7
M+E4	78	16	6
M+E5	80	13	7
M+E6	81	14	5
M+E7	78	15	7
M+E8	79	14	7
M+E9	81	16	3
**Mean(±Margin of error)	78.33(±2.25)	15.55(±2.08)	6.11(±1.55)

Table 6: The average percentage of inflammatory cells in cytological smear from lungs obtained from normal healthy, *Mycoplasma*, *E. coli* and mixed infection cases.

Groups	The average cell percentage Mean (±SD)		
	Lymphocyte (%)	Heterophil (%)	Macrophage (%)
Normal healthy (control)	77.6 ±4.06	11.5 ±1.87	10.9 ±3.48
***Mycoplasma* infected**	85.11 ±2.99	6.88 ±1.28	7.1 ±2.58
***E.coli* infected**	67.6 ±3.16	21.6 ±1.87	10.8 ±1.90
Mixed infected	78.33 ±2.25	15.55 ±2.08	6.11 ±1.55

** Margin of Error= $t_{n-1}(\frac{\alpha}{2p})\frac{SD_{ii}}{\sqrt{n}}$ where $t_{n-1}(\frac{\alpha}{2p})$ Critical Values for Bonferroni's method of multipe comparisons, *SD* refers to standard deviation, *n* refers to sample size.

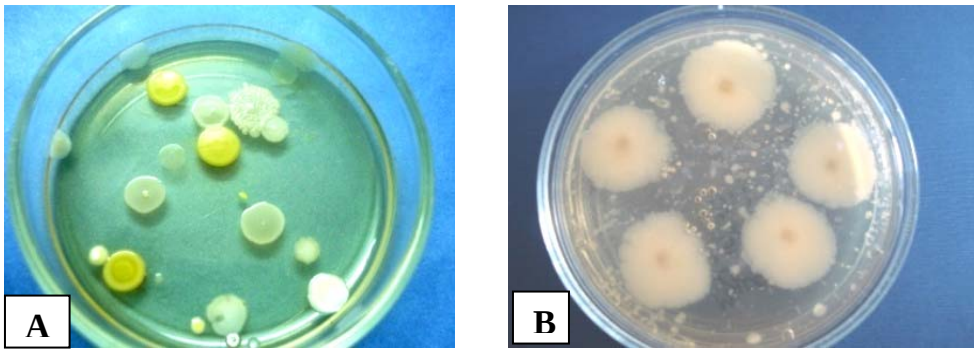

Figure 1. Growth of *Mycoplasma* supplemented with horse serum. A-B: Fried egg appearance with formation of nipple at the center, A: 8 days culture, B: 18 days culture.

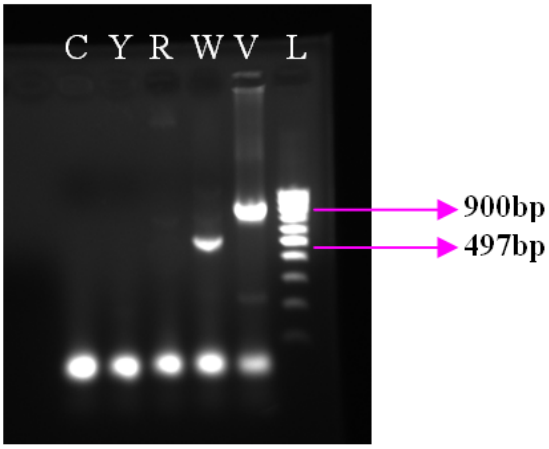

Figure 2. Electrophoresis of PCR products. L; Marker, V; Mycoplasma vaccine, W; R; Y; *Mycoplasma* samples, C; Water control. V; Vaccine (*M. gallisepticum*) and W; *M. synoviae* showed positive band 900 bp and 497 bp, respectively.

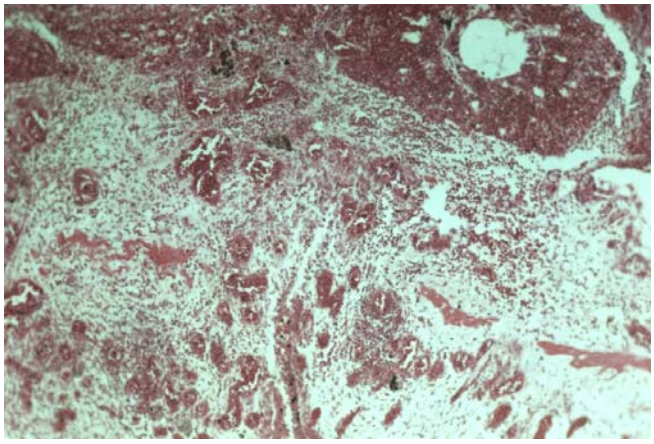

Figure 3. Pneumonia with predominant lymphocytic infiltration (H&E stain x 330).

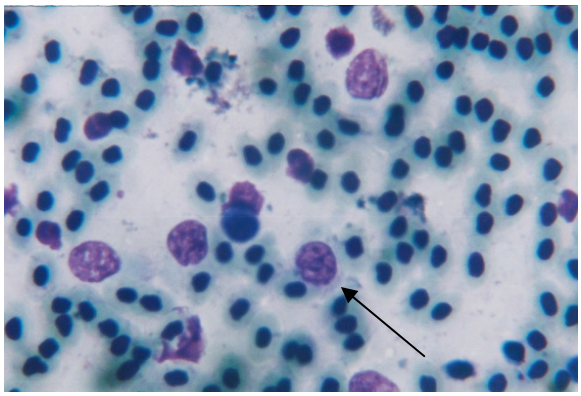

Figure 4. Lung smear from *Mycoplasma* infected bird consisting mainly lymphocyte (arrow). Giemsa stain x 825.

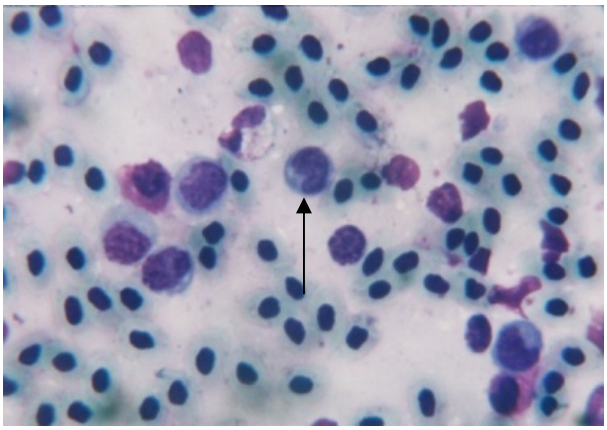

Figure 5. Lung smear from *Mycoplasma* infected cases consisting mainly macrophage (arrow), Giemsa stain, × 82.5).

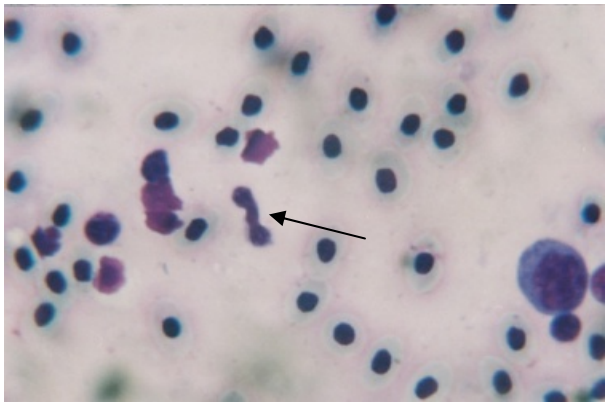

Figure 6. Lung smear from *E.coli* infected cases consisting mainly heterophil (arrow), Giemsa stain, × 82.5).