Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(6): 1623-1627

Received: 19-08-2020
Accepted: 11-10-2020

Azhaguraja Manoharan
Centre for Advanced Studies in Poultry Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Binoj Chacko
Centre for Advanced Studies in Poultry Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

P Anitha
Centre for Advanced Studies in Poultry Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Ancy M
Centre for Advanced Studies in Poultry Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Sreeveen EN
Centre for Advanced Studies in Poultry Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Vaibhav S
Centre for Advanced Studies in Poultry Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Corresponding Author:
Azhaguraja Manoharan
Centre for Advanced Studies in Poultry Science, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

A report of *Pasteurella multocida* Type A infection in an organized poultry farm in Kerala

Azhaguraja Manoharan, Binoj Chacko, P Anitha, Ancy M, Sreeveen EN and Vaibhav S

Abstract
Fowl cholera is commonly occurring bacterial disease caused by *Pasteurella multocida* (Several serotypes) which affects most of the domestic and wild birds. A confirmatory diagnosis could be done by various biochemical test and polymerase chain reaction (PCR) for serotyping the organism. The main objective of the present study was to investigate the Fowl cholera infection in an organized poultry farm in Thrissur district of Kerala. The feacal swabs, tracheal swabs and blood samples were collected and subjected to microbiological and molecular investigations. Also, these isolates were exposed to antibiotic sensitivity test (ABST) to find out the drug choice for the treatment. Our research findings confirmed that out of 28 birds of native chicken about 21 birds were found to be positive for *Pasteurella multocida* Type A infection. As pasteurellosis has a great impact on economy, early diagnosis with effective treatment and vaccination strategies should be employed in poultry.

Keywords: Fowl cholera, Pasteurella, PCR, ABST

Introduction
Pasteurella multocida type A is the causative agent of fowl cholera, a highly contagious, systemic and fatal disease of chicken [13]. Fowl cholera also known as "avian pasteurellosis" and "avian haemorrhagic septicaemia" [9]. It's a septicaemic disease which is associated with high morbidity and mortality in poultry predominantly in chicken and ducks [1]. It has great impact on poultry economy and production. Fowl cholera usually occurs either acute or chronic forms. In acute cases the clinical symptoms of fowl cholera are often occurs few hours before death which includes fever, ruffled feathers, mucus discharge from mouth, nostrils and ears and cyanosis of comb and wattles [15]. *Pasteurella multocida* is Gram negative, nonmotile, cocco-bacillus, capsulated non spore forming bacteria occurring singly, in pairs or occasionally as chains or filaments belonging to Pasteurellaceae family [13]. In general, five capsular serotypes (A, B, D, E, and F) are found in *Pasteurella multocida* and each serotype is associated with specific host. For example, serotype A and D causes fowl cholera in avian species [7]. In Asian countries, fowl cholera is caused by *Pasteurella multocida* type A: 1, A: 3 and type D. Among different types of *Pasteurella multocida*, serotype A is the most pathogenic which causes 80% mortality whereas serotype D causes 20% mortality in chickens [12]. Precise and early diagnosis are the effective methods to rule out the fowl cholera infection. A conventional methods of diagnosis is not effective and less sensitive as compared to polymerase chain reaction (PCR) technique [1]. So far in Kerala, only limited studies were carried out on molecular identification of fowl cholera in chicken. As far as we know, there is no report on identification of *Pasteurella multocida* type A in chicken using molecular technique in Kerala. Hence, the present study was aimed at isolation, identification and molecular detection of *Pasteurella multocida* type A in naturally infected chickens. In addition, antibiotic sensitivity test (ABST) has been performed to find out the specific drug of choice for the effective treatment of infected chickens.

Materials and Methods
Clinical examination
A total of 28 birds (one year old) of native chicken were showing the clinical signs of fowl cholera in an organized poultry farm, Thrissur. Upon clinical examination the infected birds were observed with pyrexia (107.6º F), greenish white diarrhoea, pale and hypertrophied comb, mucoid discharge from nostrils and swollen foot pad.

Collection of samples

The feacal swabs, tracheal swabs and blood samples (around 0.5 to 1 ml of blood from the wing vein) were collected aseptically from the infected birds in an organized poultry farm, Thrissur, Kerala. Blood smears were prepared according to the standard procedure. The collected samples were brought to laboratory for further investigation.

Isolation and identification of *Pasteurella multocida*

Pasteurella multocida organisms were cultured following the standard procedure described by [5]. The collected samples (Tracheal and feacal swabs) were streaked into different culture media namely, Blood agar (BA), Nutrient agar (NA) and Nutrient broth (NB) for the isolation of *Pasteurella multocida*. The inoculated culture plates were incubated at 37ºC for the appearance of characteristic colony. To obtain the pure culture of *Pasteurella multocida*, a selective subculture media was prepared subsequently based on the morphological characteristics of the colony. For the morphological identification of bacteria, the isolated pure culture was subjected to Grams staining. The colonies obtained on the media was subjected to biochemical characterization using various tests viz., Methyl red (MR), Voges-Proskauer (VP), Indole production, catalase, oxidase and sugar fermentation test were carried out.

Antibiotic sensitivity test (ABST)

To find out the specific drug of choice for the treatment, the isolates were subjected to ABST using different antibiotic discs employing amoxicillin, tetracycline, cephalexin, cotrimaxazole, sulphadiazine and trimethoprim, enrofloxacin and gentamicin. The zone of inhibition was measured, recorded and interpreted.

DNA extraction and polymerase chain reaction (PCR) assay

The crude DNA was extracted from the isolates using boiling method given by [18]. Polymerase chain reaction was done using specific set of forward (5′-TCCGCAGAAAATTATTGACTC-3′) and reverse primer (5′-GCTTGCTGCTTGATTTTGTC-3′) to amplify the 511bp fragment of *capE* gene of *Pasteurella multocida* type A. Each diluted primer (10 pM/µl) was added to the template DNA [working solutions prepared from stock solution by diluting with sterile distilled water (Millipore) to get a final concentration of 100 ng/µl] and 2X PCR Smart Mix (origin) in a PCR tube and made up to the final volume of 20 µl using ultra filtered Millipore water. PCR was done in Bio-Rad thermal cycler and standardization was done for each reaction by mild adjustment of concentration of ingredients and annealing temperature with the following profile: initial denaturation of 5 min at 94°C; 35 cycles of 94°C for 30 s, annealing at 55°C for 30 s, and 72°C for 30 s with a final elongation of 5 min at 72°C. PCR amplicon was subjected to 2% agarose gel.

Agarose gel electrophoresis

The genomic DNA and PCR products were checked in agarose gels of 0.8 percent and 2%, respectively prepared using 1X TBE buffer. DNA and PCR products were mixed with loading dye (6X) and loaded in wells. The PCR products were loaded along with a molecular weight marker (50bp) for relative sizing. Electrophoresis was carried out at 5V/cm until the bromophenol blue dye migrated more than two by third length of the gel and was visualized in a Gel Doc System (Bio-Rad, USA). The amplicons of *capE* gene of *Pasteurella multocida* type A (511bp) were sequenced using respective forward and reverse primers in an automated sequencer using Sanger's dideoxy chain termination method at Agri Genome Labs Pvt. Ltd., Cochin.

Results and Discussion

In the present study out of 28 birds of infected native chickens, 21 birds were found to be positive for *Pasteurella multocida*. On Geimsa staining, blood smears revealed the presence of bipolar organisms (Fig.1). In comparison, [21] observed numerous bipolar organisms characteristic of *Pasteurella* spp. upon microscopic examination of Geimsa stained smears of heart blood in water fowl. The clinical samples collected during an outbreak of heamorrhagic septicaemia in a buffalo farm of West Bengal, India. On microscopic observation, peripheral, heart blood smear and tissue impressions revealed the presence of Gram negative, bipolar cocco – bacilli organisms alike of *Pasteurella* spp. [11]

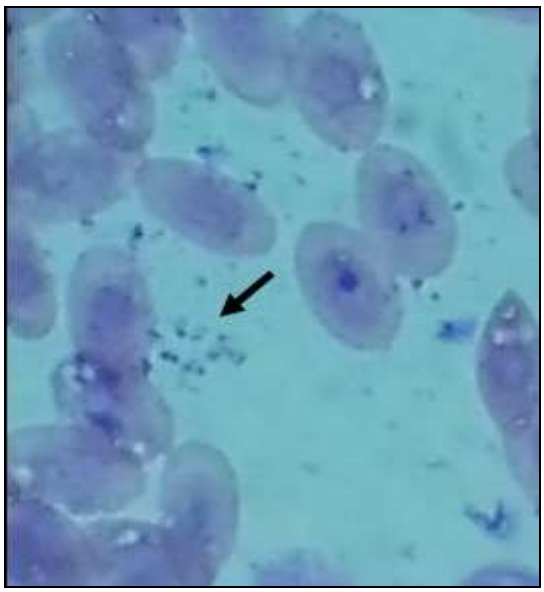

Fig 1: Bipolar appearance of *Pasteurella multocida* in Geimsa staining

Cultural examination of tracheal and feacal swabs revealed the presence of small, glistening, mucoid, dew drop like colonies on blood agar (BA) after an overnight incubation (Fig.2). [16] Reported the same colony morphology in the samples which are collected from private poultry farm, Maharastra during the Fowl cholera outbreak. Similar findings was reported by [2] in cattle and buffalo of Karachi region of Pakistan. Also, [8, 13] reported the indistinguishable findings in native chicken of Bangladesh. In addition, the recovered bacteriological isolates were shown smooth, glistening and translucent colonies on blood agar and produced non-heamolytic dewdrop colonies on sheep blood agar. However, these isolates were failed to grow on McConkey's agar [11]. These findings were accordance with that of [6] who conducted a research on isolation, identification and antimicrobial susceptibility patterns of *Pasteurella multocida* isolated from diseased rabbits. As stated by [3], a few strains of Fowl cholera organisms may not grow in media without blood or blood serum. In the present study, the selected isolates were found to grow well in bovine blood agar media.

Fig 2: Small, glistening, mucoid, dew drop like colonies on blood agar (BA) media

The selected isolates were found be to Gram-negative, cocco-bacillary shaped *Pasteurella multocida* on Gram staining (Fig. 3). Similarly, the microscopical examination of gram stained smears of suspected isolates revealed characteristic Gram-negative, non-spore forming and cocco-bacillary organisms arranging singly, in pairs or short in chains. Furthermore, a typical bipolar staining rods were evident in Giemsa stained smears of Pasteurella fresh cultures [6]. These results were accordance with that of [2, 8, 13, 1]. Also, [11] reported the indistinguishable findings from the collected samples suspected of Haemorrhagic septiceamia in an organized buffalo farm.

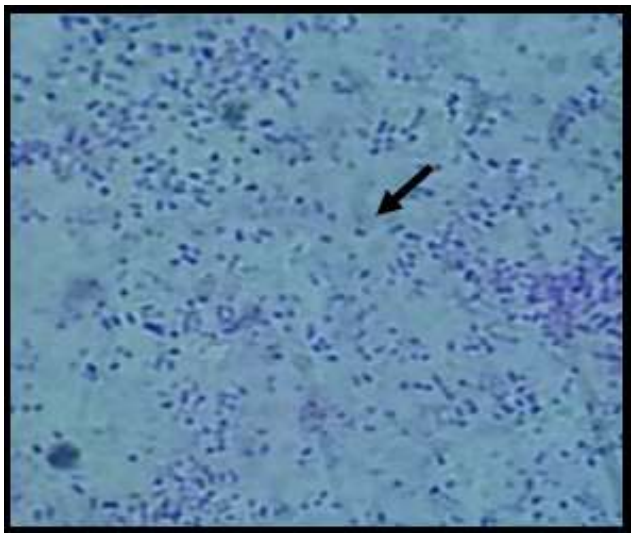

Fig 3: Gram negative, cocco bacillary or rod shaped *P. multocida* on Gram staining

On biochemical characterization, *Pasteurella multocida* organism were shown to be positive for catalase and oxidase test (Fig.4 and 5). On the contrary, Methyl red (MR) and Voges-Proskauer (VP) test did not show any reaction. Thus, *Pasteurella multocida* organism is negative for MR and VP tests. Sugars such as dextrose, sucrose and mannitol were fermented by *Pasteurella multocida* organism whereas maltose and lactose was not fermented. In comparison, these findings of biochemical reactions were similar with that of [18, 19, 8, 4 13].

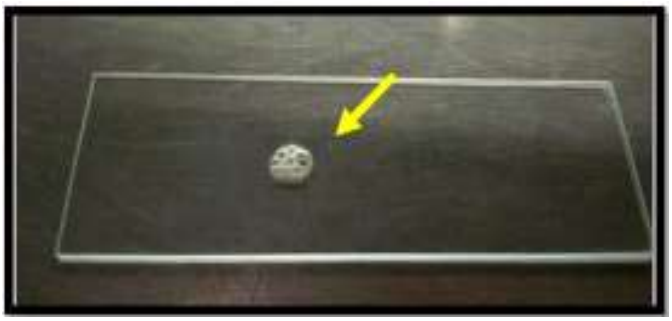

Fig 4: Catalase test of *Pasteurella multocida* showing positive reaction

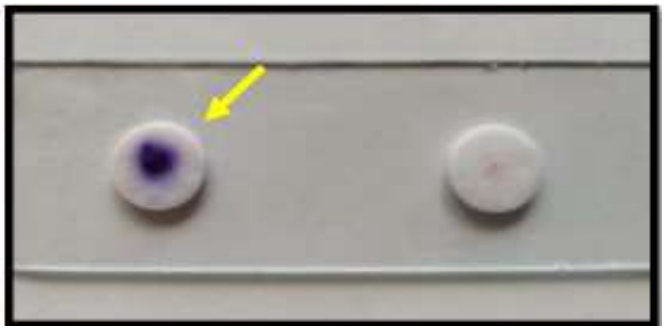

Fig 5: Oxidase test of *Pasteurella multocida* showing positive reaction

The isolates were found to be sensitive to sulphadiazine and trimethoprim and cephalexin, and resistant to amoxicillin, tetracycline, cotrimaxazole, enrofloxacin and gentamicin (Fig.6). In contrast, [16, 14] were reported that isolates of *Pasteurella multocida* were sensitive to enrofloxacin, gentamycin and enroflaxacin, cephalexin in chicken and Pigeon, respectively. On the basis of ABST results, infected birds were treated with Injection Biotrim (Sulphadiazine and trimethoprim) at a dose rate of 25mg/Kg bodyweight intramuscularly and Cephalex powder (Cephalexin) at the rate of 0.3gm/ day orally for one week. During the course of treatment, about 3 birds were died out of 21. The remaining 18 birds were uneventfully recovered after one week of post treatment. Blood smear taken after a week was negative *Pasteurella* organisms.

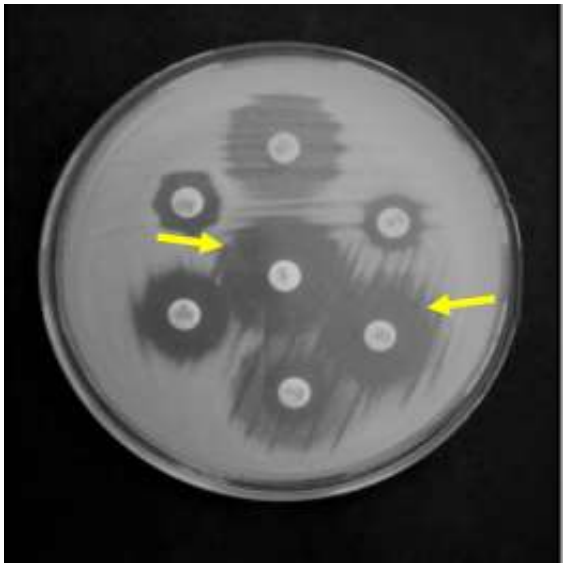

Fig 6: ABST – Isolates were found sensitive to sulphadiazine and trimethoprim and cephalexin

In addition, for the identification and molecular characterization of *Pasteurella* spp., the isolated samples were subjected to polymerase chain reaction (PCR) with specific set of primers. The PCR assay revealed the 511bp amplicon fragment of *capE* gene of *Pasteurella multocida* type A (Fig. 7). In comparison, [13] carried a research on isolation and molecular detection of *Pasteurella multocida* type A in naturally infected chicken in Bangladesh. Also, [1] reported the similar findings in which out five dead chickens two were found to be positive for *Pasteurella multocida* type A infection in brother's poultry farm located at Konabari, Gazipur, Bangladesh. Similarly, [10] conducted a research on prevalent serotypes of *Pasteurella multocida* isolated from different avian species of India and found that majority of avian isolates were belonged to serotype A. On the other hand, [17] reported that prevalence of *Pasteurella multocida* capsular serotype B was found to be high in cattle and buffaloes of Iran. In case of ruminants, *Pasteurella multocida* serotype B causes Haemorrhagic septicaemia (HS) with high mortality and buffaloes are more susceptible than cattle. In Kerala, infection of *Pasteurella multocida* in poultry has been reported in many places but there were no reports have been made on molecular identification of *Pasteurella multocida* type A. The research findings of our present study reported the successful molecular diagnosis of *Pasteurella multocida* type A in naturally infected native chickens of Kerala. These results suggest that PCR assay could be an effective tool for the early diagnosis of disease. Also, PCR is comparatively more sensitive than conventional methods of diagnosis in order to identify the specific serotype of a particular organism. Hence PCR technique can be successfully applied to detect the different serotypes of *Pasteurella multocida* in poultry and other species.

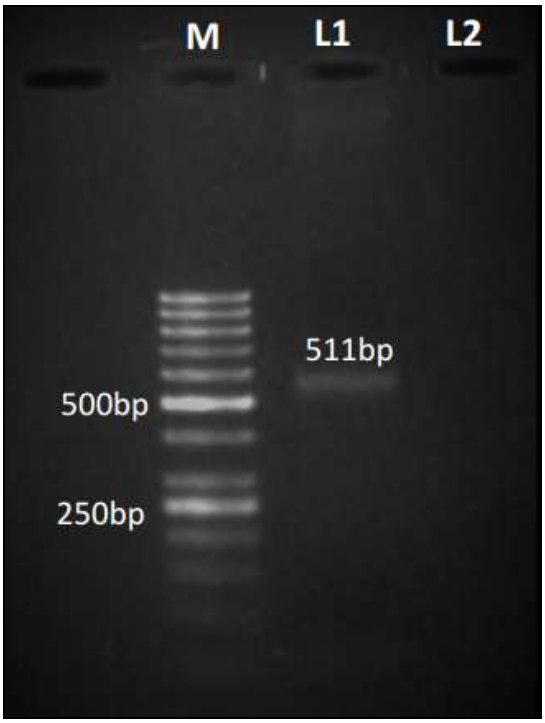

Fig 7: Amplification of 511 fragment of capE gene of *Pasteurella multocida* type A. M- Marker, L1- Sample, L2- Negative control

Conclusion

In this study, a successful isolation and molecular characterization of *Pasteurella multocida* type A from naturally infected native chickens was carried out using PCR assay. Since the bacterial isolation and identification are time consuming procedures, PCR could be an effective tool for timely diagnosis of the disease. Also, Pasteurellosis has great impact on poultry economy, early diagnosis with effective treatment and vaccination strategies should be implemented. Thus, molecular identification of fowl cholera could be an efficient method of diagnosis in future studies.

Acknowledgment

I would like to thank the senior scientist i/c of AICRP on Poultry improvement and the Director, Centre for Advanced Studies in Poultry Science and the Dean, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala for providing the necessary facilities needed for the present study.

References

1. Akhtar M, Rahman MT, Ara MS, Rahman M, Nazir KNH, Ahmed S *et al*. Isolation of *Pasteurella multocida* from chickens, preparation of formalin killed fowl cholera vaccine, and determination of efficacy in experimental chickens. Journal of Advanced Veterinary and Animal Research 2016;3(1):45-50.
2. Ashraf A, Tariq H, Shah S, Nadeem S, Manzoor I, Ali S *et al*. Characterization of *Pasteurella multocida* strains isolated from cattle and buffaloes in Karachi, Pakistan. African journal of microbiology research 2011;5(26):4673-4677.
3. Carter GR. Improved hemagglutination test for identifying type A strains of Pasteurella multocida. Applied microbiology 1972;24(1):162-163.
4. Christensen H, Nicklas W, Bisgaard M. Investigation of taxa of the family Pasteurellaceae isolated from Syrian and European hamsters and proposal of Mesocricetibacter intestinalis gen. nov., sp. nov. and Cricetibacter osteomyelitidis gen. nov., sp. nov. International journal of systematic and evolutionary microbiology 2014;64(11):636-3643.
5. Cowan ST. Cowan and Steel's manual for the identification of medical bacteria. Cambridge university press, Cambridge, London 2004, 122-125.
6. El Sayed M, El-Mowalid G, Ali A, Arnaout M, Abd El Hamid M. Isolation, Identification and Antimicrobial susceptibility patterns of Pasteurella multocida Isolated from Diseased Rabbits. Suez Canal Veterinary Medical Journal. SCVMJ 2018;23(1):13-26.
7. Harper M, Boyce JD, Adler B. *Pasteurella multocida* pathogenesis: 125 years after Pasteur. FEMS microbiology letters 2006;265(1):1-10.
8. Ievy S, Khan MFR, Islam MA, Rahman MB. Isolation and identification of *Pasteurella multocida* from chicken for the preparation of oil adjuvanted vaccine. Microbes and Health 2013;2(1):1-4.
9. Johnson TJ, Abrahante JE, Hunter SS, Hauglund M, Tatum FM, Maheswaran SK *et al*. Comparative genome analysis of an avirulent and two virulent strains of avian *Pasteurella multocida* reveals candidate genes involved in fitness and pathogenicity. BMC microbiology 2013;13(1):106.
10. Kumar AA, Shivachandra SB, Biswas A, Singh VP, Singh VP, Srivastava SK. Prevalent serotypes of Pasteurella multocida isolated from different animal and avian species in India. Veterinary research communications 2004;28(8):657-667.

11. Mohamed MA, Mohamed MW, Ahmed AI, Ibrahim AA, Ahmed MS. *Pasteurella multocida* in backyard chickens in Upper Egypt: incidence with polymerase chain reaction analysis for capsule type, virulence in chicken embryos and antimicrobial resistance. Veterinaria Italiana 2012;48(1):77-86.
12. Panna SN, Nazir KNH, Rahman MB, Ahamed S, Saroare MG, Chakma S *et al*. Isolation and molecular detection of *Pasteurella multocida* Type A from naturally infected chickens, and their histopathological evaluation in artificially infected chickens in Bangladesh. Journal of Advanced Veterinary and Animal Research 2015;2(3):338-345.
13. Ramesh S, Soundararajan C, Manimaran K, Subapriya S, Sokkalingam R. Incidence of Pasteurellosis in a Pigeon (Columba livia)-A Case Report. International Journal of Current Microbiology and Applied Sciences 2018;7(12):3693-3697.
14. Saif YM, Barnes H, Glisson JR, Fadly AM, McDougald LR, Swayne DE. Diseases of poultry. 12. Ames, Iowa: Blackwell Pub Professional 2008, 452-514.
15. Sarangi LN, Panda HK. Antibiotic Sensitivity of Avian Isolates of *Pasteurella multocida*. Indian Veterinary Journal 2011;88(6):85-86.
16. Shayegh J, Atashpaz S, Salehi TZ, Hejazi MS. Potential of *Pasteurella multocida* isolated from healthy and disease cattle. Bulletin of the Veterinary Institute Pulawy. 2010;54:299-304.
17. Shivachandra SB, Kumar AA, Gautam R, Singh VP, Saxena MK, Srivastava SK. Identification of avian strains of *Pasteurella multocida* in India by conventional and PCR assays. The Veterinary Journal 2006;172(3):561-564.
18. Tabatabai LB. Identification of *Pasteurella multocida* CHAPS-soluble outer membrane proteins. Avian diseases 2008;52(1):147-149.
19. Queipo-Ortuño MI, Colmenero JDD, Macias M, Bravo MJ, Morata P. Preparation of bacterial DNA template by boiling and effect of immunoglobulin G as an inhibitor in real-time PCR for serum samples from patients with brucellosis. Clinical and Vaccine Immunology 2008;15(2):293-296.
20. Vaught RW, McDougle HC, Burgess HH. Fowl cholera in waterfowl at Squaw Creek National Wildlife Refuge, Missouri. The Journal of Wildlife Management 1967;31(2):248-253.