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Immune-histochemistry and molecular characterization techniques for detection of *Pasteurella spp* virulence genes in cattle

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ABSTRACT

Fifty three pneumonic cattle lung of both sexes were sampled. Fourteen bacterial isolates (12 *P. multocida* and 2 *M. haemolytica*) were positively approved from lung tissue. Isolation occurred by ordinary colonial and biochemical confirmation. cultural characteristics were identified on to blood agar from brain heart infusion broth by ordinary methods. Isolates harbored omp87, nanB and toxA virulence genes (75%, 58.3% and 66.7%) for *P. multocida*, while lktC virulence genes (100%) were determined in all of tested *M. haemolytica* isolates. Histopathology of the examined lungs were performed and the microscopical changes were described. Immunohistochemical staining of the positive reactors reveal significant increase of CD68 cells in *P. multocida* and *M. haemolytica* affected tissues; Chronic interstitial pneumonia (*P. multocida*), Fibrinous pneumonia (*P. multocida*), chronic bronchopneumonia (*P. multocida*) and Chronic pneumonia (*M. haemolytica*) (2.66, 4.43, 5.40 and 6.21) respectively. In summary, detection of virulence genes is important for more controllable method of the pathogen and CD68 cell IHC tool approved its ability pathologically for confirmatory diagnosis of inflammation and pathological alterations exhibited by *P. multocida* and *M. haemolytica* pathogens.

INTRODUCTION:

Pasteurella spp of the Pasteurellaceae tree has different disease synonyms in many animal species as shipping fever in small ruminants, haemorrhagic septicaemia in large ruminants, avian pasteurellosis in fowls and snuffles in

rabbits (Harper et al. 2006 and Dziva et al. 2008). Vaccination was the major protective protocols against this diseases but some negatives were obtained about this vaccine; the serious one is short-acting immunization (Shivachandra et al. 2011 and Verma et al.

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1998). The vaccination procedure depends upon mainly the outer membrane proteins (OMPs) as an antigenic particle (Shivachandra et al. 2011).

In septicemic pasteurellosis, *P. multocida* is likely transmitted between cattle by ingestion or inhalation during direct contact with an affected animal or contaminated feed or water. However, it is also believed that *P. multocida* can survive for hours or days in decomposing carcasses and in damp soil or water, thus contact with these infected materials could represent another possible route of infection (De Alwis 1999). *Pasteurella multocida* can reside in the tonsils of the nasopharynx of apparently healthy cattle and buffalo; therefore, some species may act as a reservoir of bacteria that cause septicemic pasteurellosis (De Alwis et al. 1990).

Hemorrhagic septicemia is a clinically indistinguishable disease that occurs in domestic cattle (*Bos taurus*) and water buffalo (*Bubalis bubalis*) and is enzootic to tropical countries in Africa and Asia. Hemorrhagic septicemia is most commonly caused by *P. multocida* serotypes B:2 and E:2, but serotypes A:1, A:1,3, A:3, A:4, B:1, B:2,5, B:3,4, E:2,5, F:3, F:3,4 have also been implicated (Shivachandra et al. 2011).

The clinical signs of septicemic pasteurellosis include increased body temperature, loss of appetite, depression, excessive salivation, edema of the head, neck, and brisket, and severe respiratory distress with foamy nasal discharge, leading to death (Shivachandra et al. 2011 and De Alwis. 1999) The disease occurs in acute, sub-acute, and chronic forms, and in the acute form of septicemic pasteurellosis death will occur in less than 24 h (Shivachandra et al. 2011).

At necropsy, the carcass was in good nutritional condition with no evidence of dehydration and moderate autolysis. There was extensive subcutaneous edema over the right lateral thorax and abdomen. There was marked subcutaneous edema extending over the proximal aspect of the right hind limb. On section, the

gracilis and semitendinosus muscles were dark red and dry, and the fascia was expanded by edema, hemorrhage, and fibrin consistent with necrotizing and hemorrhagic myositis. Post-mortem examination revealed necrotizing and hemorrhagic myositis, fibrinous pericarditis and multi-systemic bacterial emboli (Douglas D-B et al. 2020).

At necropsy, some of the most common gross pathological findings are subcutaneous edema in the mandibular and brisket regions, pericarditis, and widespread petechiae to ecchymoses (De Alwis. 1999 and Rhoades KR et al. 1967). Common histopathological changes include thickened alveolar septa of the lungs with increased lymphocytes and macrophages, swelling and pyknosis of tubular epithelial cells of the kidney, and subepicardial and sub-endocardial hemorrhages of the heart (Rhoades KR et al. 1967). However, gross pathological and histopathological findings can differ depending on the duration of the disease from acute onset to death (De Alwis. 1999).

Characteristic gross lesions founded were reddening coloration and firm to hard consolidation; may have irregularly shaped non-friable foci of coagulation necrosis, interlobular edema (marbling appearance), or fibrinous pleuritis (Jeff et al. 2012).

Pasteurella pathogen cannot cause disease without their virulent factors, fimbriae and adhesions (nan B and nanH), a variety of many outer membrane proteins (OMPs) like protectins (ompA, ompH, omp87 and plpB), and dermonecrotxin (toxA) are the most important ones (Jamali et al. 2014) The mode of action of these genes to destroy the affected cells via colonization, toxin secretion and host inflammation stimulation, hence immune impairment (Harper et al. 2006).

These OMPs contain important virulence genes; main factors for intraspecies diversity (Davies et al. 2004) and immunological vaccinal components (Dabo et al. 1997). CD68 is heavily glycosylated glycoprotein that is highly expressed in macrophages and other mononuclear phagocytes and used for histochemical analysis of inflamed tissue and bacterial lipo-

polysaccharide. The purpose of this study is to detect the bacterial virulence genes of *Pasteurella spp* by the methods of PCR and immunohistochemistry in cattle.

MATERIALS and METHODS

Sampling and Bacterial Isolation and Identification:

A total of 53 pneumonic cattle lung of both sexes were sampled. All lung during the period from January 2022 to December 2022. Fourteen bacterial isolates (12 *P. multocida* and 2 *M. haemolytica*) were positively approved from lung tissue. Isolation occurred by ordinary colonial and biochemical confirmation (El-Seedy et al. 2020). Maintained at Department of food hygiene, faculty of veterinary medicine, Assiut, Egypt, were used.

Confirmation of *P. multocida* Isolates:

The isolates were revived in brain heart infusion broth by 18–24 h incubation at 37°C

and plated subsequently onto blood agar to study cultural characteristics. The cultures were then tested for purity by biochemical tests as per standard techniques (Cowan and Steel, 1970).

Detection of Virulence Associated Genes by PCR:

PCR was applied to determine the *nanB*, *omp87* and *toxA* virulence-associated genes in *P. multocida* isolates and *lktC* virulence-associated genes in *M. haemolytica* isolates. Primers sequences and amplified products for the targeted genes for *P. multocida* and *M. haemolytica* isolates are illustrated in Table 1.

Table 1. Primers sequences and amplified products for the targeted genes for *P. multocida* and *M. haemolytica* isolates:

Bacteria	Target gen	Primer sequence 5'-3'	Amplified product	Reference
<i>P. multocida</i>	<i>omp87 F</i>	AGGTGAAAGAGGTTATG	200 bp	Sabiel et al. 2012
<i>P. multocida</i>	<i>omp87 R</i>	TACCTAA CTCAACCAAC	200 bp	Sabiel et al. 2012
<i>P. multocida</i>	<i>nanBF</i>	AGGTGAAAGAGGTT ATG	554 bp	Sarangi et al. 2014
<i>P. multocida</i>	<i>nanBR</i>	ACAGCAAAGGAAGACTGTCC	554 bp	Sarangi et al. 2014
<i>P. multocida</i>	<i>toxAF</i>	CTTAGATGAGCGACAAGG	864 bp	Tang et al. 2009
<i>P. multocida</i>	<i>toxAR</i>	GAATGCCACACCTCTATAG	864 bp	Tang et al. 2009
<i>M. haemolytica</i>	<i>lktCF</i>	GGAAACATTACTTGGCTATGG	440 bp	Klima et al. 2014
<i>M. haemolytica</i>	<i>lktCR</i>	TGTTGCCAGCTCTTCTTGATA	440 bp	Klima et al. 2014

Gene Sequencing and Sequence Analysis

Due to high cost, sequencing of *toxA* gene of one strain of *P. multocida* serotype B:2 as well as *lktC* gene of one strain of *M. haemolytica* serotype 2 was applied according to the method of Tamura et al. (2013).

Immunohistochemistry

Sections of lung were prepared for Immunohistochemical analysis using an Ultra Tek HRP anti-polyvalent (DAB) staining system (ScyTek Laboratories, West Logan, UT, USA, AMF080). The sections were deparaffinized with xylene, rehydrated in graded ethanol, and

washed with distilled water. The sections were heated for 10 min in asodium citrate buffer (0.01 M, pH 6.0) to increase epitope exposure. The sections were cooled at room temperature (RT) for 30 min and washed with PBS. The endogenous peroxidase activity was quenched with 3% H₂O₂ in distilled water for 15 min at RT followed by washing with PBS (2X5 min). The sections were blocked with the blocking solution of the kit for 5 min at RT. The sections were incubated overnight at 4 C° with mouse monoclonal anti-CD68 (1:100); Santa Cruz, sc-17832). Sections were rinsed (three times and 5 min each) with PBS and were incubated for 15 min with the secondary Ultra Tek HRP Anti-polyvalent antibody (goat anti-mouse, rat, rabbit and guinea pig IgG) purchased from Scy Tek, (TX, USA). Following that, the slides were washed three times for 3 min each with a wash buffer. The visualization of the reaction was carried out with Diaminobenzidine (DAB) chromogen diluted with DAB substrate (provided within the same Scy Te Detection kit) according to the manufacturer protocol for 10-15 min until the desired staining was achieved and counterstained with Harris hematoxylin and mounted with mounting media, DPX. The number of immunopositive cells was counted in 5 separate microscop-

ic fields in each slide and the mean number for each slide was obtained, then the mean \pm SE was calculated for each group.

Histopathology:

Tissue samples from lung were fixed in 10 % neutral buffered formalin. They were dehydrated by ascending grades of alcohol, cleared by xylene and embedded in paraffin. Sectioning of the tissue with 5 microns thickness and stained with hematoxylin and eosin (H&E) (Bancroft et al. 1996).

Statistical analysis:

Statistical analysis of the data generated from the study was performed with Graphpad prism 5. *P* values of <0.05 were considered as statistically significant.

RESULTS

Detection of virulence genes of *P. multocida* and *M. haemolytica* Isolates by PCR:

Our finding of PCR exhibits that eight (75%, 58.3% and 66.7%) *P. multocida* isolates and two (100%) *M. haemolytica* isolates were positive for *Omp87*, *nanB*, *toxA* and *IktC* genes, respectively (Table 2).

Table 2. Percentage of virulence genes of *P. multocida* and *M. haemolytica* isolates:

Tested Bacteria	Tested Gene	No. of the tested Isolates	Positive		Negative	
			NO.	%	No.	%
<i>P. multocida</i>	<i>Omp87</i>	12	9	75	3	25
	<i>nanB</i>		7	58.3	5	41.7
	<i>toxA</i>		8	66.7	4	33.3
<i>M. haemolytica</i>	<i>IktC</i>	2	2	100	0	0

Immunohistochemistry results:

Immunohistochemical staining reveal significant increase of CD68 cells in *P. multocida*

and *M. haemolytica* affected tissues than normal lung (table 3).

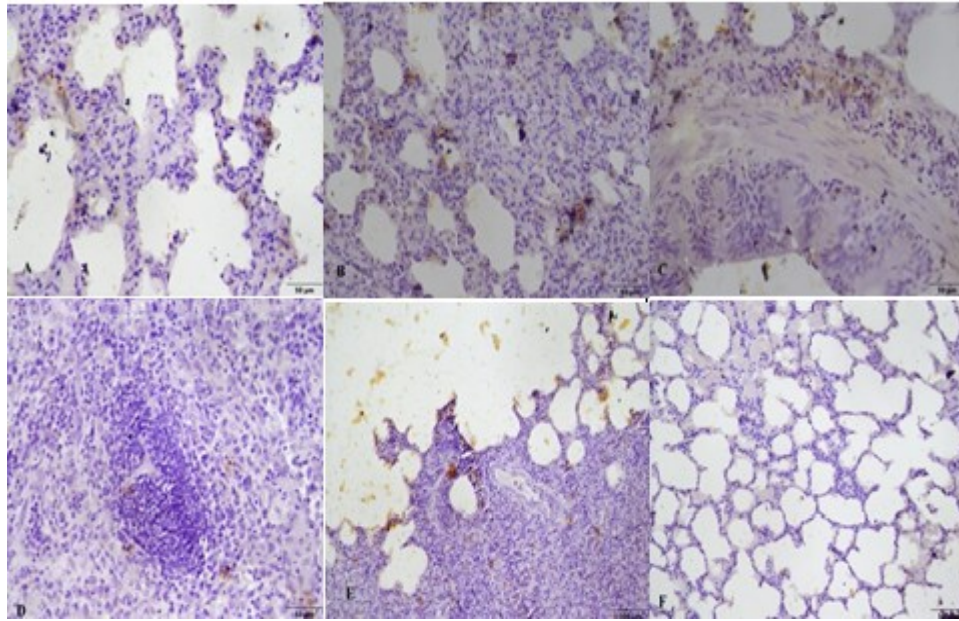


Fig 1. A-E) Scale bar A,C and D = 50, Scale bar B and E = 100 μ m., Lung showing prevalence of specific Immunohistochemical reaction of CD68 cells against *Pasteurella multocida* and *Manheimia haemolytica*. F) Scale bar = 100 μ m, Normal lung showing absence of CD68 cells and no rod shaped bacteria..

Histopathological Results:

Microscopically, of the cattle lungs examined There was Chronic Interstitial pneumonia, fibrinous pneumonia, chronic bronchopneumonia and Chronic pneumonia. **chronic interstitial pneumonia exhibited** diffuse thickening of the interstitium due to fibrosis, increased cellularity and smooth musculature. while the characteristic feature of **fibrinous pneumonia**

is fibrinous exudate with erythrocytic aggregation in the alveoli and bronchiole. **chronic bronchopneumonia** showed Fibrosis and hyperplasia with moderate cellular exudate in the alveoli and bronchium. **chronic pneumonia** with high aggregation of mononuclear cells and loss architecture of lung tissue.

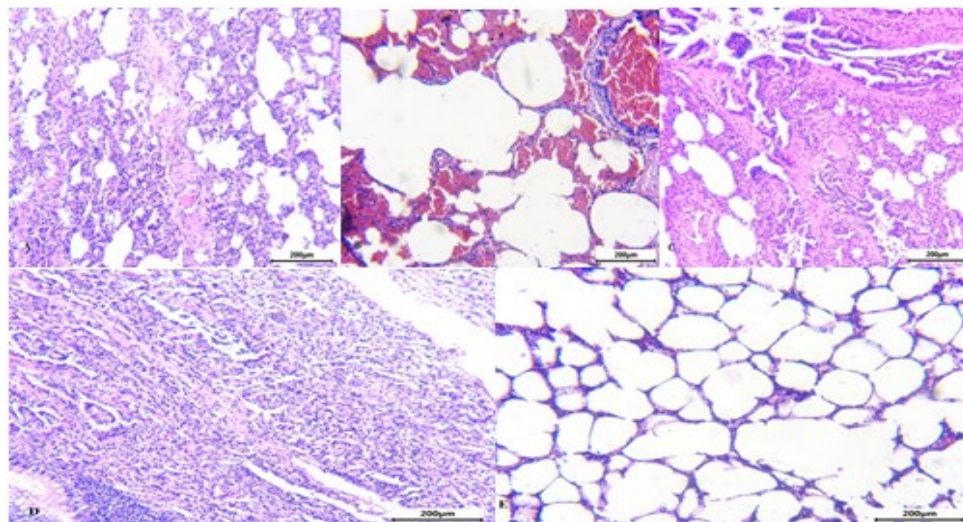


Fig 2. A-E) H&E staining in lung of different affected groups Scale bar = 200 μ m A) chronic interstitial pneumonia with marked thickening of the interstitium and diffuse aggregation of mononuclear cells. B) fibrinous pneumonia with marked fibrinous exudate within alveoli and bronchiole. C) chronic bronchopneumonia showed Fibrosis and hyperplasia with moderate cellular exudate in the alveoli and bronchium. D) chronic pneumonia with high aggregation of mononuclear cells and loss architecture of lung tissue. E) normal alveoli of lung tissue.

Table 3. Effect of different pathological affections on the percentage expression of CD68 in different *Pasteurella multocida* and *Manhemia haemolytica* infection of bovine lungs:

Group	A: Chronic interstitial pneumonia (<i>P. multocida</i>)	B: Fibrinous pneumonia (<i>P. multocida</i>)	C: chronic broncho-pneumonia (<i>P. multocida</i>)	D, E: Chronic pneumonia (<i>M. haemolytica</i>)	Control -ve
Immune stain					
CD68	2.66 ^c	4.43 ^b	5.40 ^b	6.21 ^b	N ^a

^{a, b} values are not sharing a common superscript letter differ significantly at $P < 0.05$.

DISCUSSION:

The diversity of various virulence genes of *Pasteurella multocida* and *Manhemia haemolytica* is the main forth to fight against animal species they live. Therefore, our research microbiologically focusing on detection of these virulence in an impact to counteract these harmful pathogens in future (Sarangi et al. 2014). Two of the virulent genes are detected (nanB and Omp87); their effect depends mainly on the discovery of key host receptor, inhibition of mucin release and bacterial colony formation (Hatfaludi et al. 2010). While toxA gene is the main indicator of pneumonia caused by *P. multocida* isolates (Harper et al. 2006). Furthermore, toxA (Dermonecrotxin gene) is mainly related to cattle and porcine species (Hatfaludi et al. 2010). *M. haemolytica* has many leukotoxins; the main one is lktC as the activator for leukotoxin (lktA) playing the same role of *P. multocida* virulence genes, the induction of inflammation as a consequence of leukocyte lysis as well (Singh et al. 2011 and Highlander et al. 1990). Our study confirms the previous reports.

The old method of the culture considers one of the shiny methodology continued nowadays for *Pasteurella spp* identification; although time consuming and specific transportable media were main drawbacks that face microbiologists needs (Townsend et al. 2000).

Though PCR with its advantages (more fastness, more sensitive and more specific) has the ability to overcome the ordinary method, Pathologists have a realistic, low cost and confirmatory method for *Pasteurella sp* identifica-

tion by Immunohistochemistry technique (IHC) (Kılıç et al. 2004). One of accurate reliable IHC technique is CD68 Cells. CD68 is mainly composed of glycogen and amino-acids type I endosomal/lysosomal compound (Holness and Simmons 1993). CD68 shares similar structures with lysosomal-associated membrane proteins (LAMPs) and belongs to the LAMP family of glycoproteins (Holness et al. 1993). The main action of CD68 is mainly intrapeptide for antigen expression on the surface membrane (Barois et al. 2002). CD68-positive reactivity was nearly negative in healthy tissues (Amanzada et al. 2013).

The first line of immune defense is macrophages, macrophages have on their surface Scavenger Receptors (SRs) (Penberthy and Ravichandran, 2016). CD68 is an important Scavenger type D (SCARD) belonging to SRs group (Moore and Freeman, 2006). Furthermore, the main stimulatory receptor to inflammation and bacterial lipopolysaccharide (LPS) on macrophage surface is CD68 (O'Reilly et al. 2003). Accordingly, our Immunohistochemical analysis revealed high prevalence of CD68-positive reactive cells against *Pasteurella multocida* and *Manhemia haemolytica*.

CONCLUSION

To sum up, it is important to detect some virulence-associated genes of *P. multocida* and *M. haemolytica* in our citizen. As it aid in developing a suitable control methods. Pathologically, CD68 is a new accurate detectable tool for diagnosis of *P. multocida* and *M. haemolytica* as it is more cost saver, accurate and confirmatory than ordinary culture.

REFERENCES

- Amanzada A, Malik IA, Blaschke M. 2013. Identification of CD68(+) neutrophil granulocytes in vitro model of acute inflammation and inflammatory bowel disease. *Int J Clin Exp Pathol.* (6):561–570.
- Banchroft JD, Stevens A, Turner DR. 1996. *Theory and practice of histological techniques.* Fourth Ed. Churchill Livingstone, New York, London, San Francisco, Tokyo.
- Barois N, de Saint-Vis B, Lebecque S. 2002. MHC class II compartments in human dendritic cells undergo profound structural changes upon activation. *Traffic.* (3):894–905.
- Cowan ST, Steel KJ. 1970. *Manual for the Identification of Medical Bacteria,* Cambridge University Press, Cambridge, UK.
- Dabo SM, Confer AW, GL. 1997. Murphy, “Outer membrane proteins of bovine *Pasteurella multocida* serogroup A isolates. *Veterinary Microbiology.* 54(2):167–183.
- Davies RL, Mac Corquodale R, Reilly S. 2004. Characterisation of bovine strains of *Pasteurella multocida* and comparison with isolates of avian, ovine and porcine origin. *Veterinary Microbiology.* 99(2): 145–158.
- Douglas DB, Musangu N, Eugene J, Robert EB, Jennifer LD. 2020. Septicemic pasteurellosis causing peracute death and necrotizing myositis in a beef heifer calf (*Bos taurus*) in Alberta, Canada. *The Canadian veterinary journal.* 61(12): 2020.
- Dziva, F, Muhairwa AP, Bisgaard M, Christensen H. 2008. Diagnostic and typing options for investigating diseases associated with *Pasteurella multocida*. *Veterinary Microbiology.* 128(1-2): 1–22.
- De Alwis. 1999. MC. *Haemorrhagic septicaemia.* Canberra, Australia: Australian Centre for International Agricultural Research; Contract No.: ACIAR Monograph No. 57.
- De Alwis M, Wijewardana TG, Gomis AI, Vipulasiri A. 1990. Persistence of the carrier status in haemorrhagic septicaemia (*Pasteurella multocida* serotype 6: B infection) in buffaloes. *Trop Anim Health Prod.* (22):185–194.
- El-Seedy FR, Hassan HM, Nabih AM, Salem SE, Khalifa E, Menshawy AMS, Abed AH. 2020. Respiratory affections in calves in upper and middle Egypt: Bacteriologic, immunologic and epidemiologic studies. *Adv. Anim. Vet. Sci.* (8): 558–569.
- Harper M, Boyce JD, Adler B. 2006. *Pasteurella multocida* pathogenesis: 125 years after Pasteur. *FEMS Microbiology Letters.* 265(1): 1–10.
- Hatfaludi T, Al-Hasani K, Boyce JD, Adler B. 2010. Outer membrane proteins of *Pasteurella multocida*. *Veterinary Microbiology.* 144 (1-2): 1–17.
- Highlander SK, Engler MJ, Weinstock GM. 1990. Secretion and expression of the *Pasteurella haemolytica* Leukotoxin. *J. Bacteriol.* (172): 2343–2350.
- Holness CL, da Silva RP, Fawcett J. 1993. Macrosialin, a mouse macrophage-restricted glycoprotein, is a member of the lamp/lgp family. *J Biol Chem.* (268):9661–9666.
- Holness CL, Simmons DL. 1993. Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. *Blood.* (81):1607–1613.
- Jamali H, Rezagholipour M, Fallah S, Dadrasnia A, Chelliah S, Velappan RD, Wei KSC, Ismail S. 2014. Prevalence, characterization and antibiotic resistance of *Pasteurella multocida* isolated from bovine respiratory infection. *Vet. J.* (202): 381–383.
- Jeff LC, Joanne H, Đurda S, Josepha D, Ken B. 2012. Laboratory and Postmortem Diagnosis of Bovine Respiratory Disease *Vet Clin North Am Food Anim Pract.* 28 (3): 419–441.
- Kılıç A, Muz A, Pnomonili sığır akciğerlerinden bakteri izolasyonları ve izole. 2004. *Pasteurella*’ların Polimeraz Zincir Reaksiyonu ile saptanması. *Turk J Vet Anim Sci.* (28): 217-223.
- Klima, CL, Alexander TW, Hendrick S, McAl-

- lister TA. 2014. Characterization of *Mannheimia haemolytica* isolated from feedlot cattle that were healthy or treated for bovine respiratory disease. *Canad. J. Vet. Res.*(78): 38–45.
- Moore KJ, Freeman MW. 2006. Scavenger receptors in atherosclerosis: beyond lipid uptake. *Arterioscler Thromb Vasc Biol.* (26): 1702–1711.
- O'Reilly D, Quinn CM, El-Shanawany T. 2003. Multiple Ets factors and interferon regulatory factor-4 modulate CD68 expression in a cell type-specific manner. *J Biol Chem.* (278):21909–21919.
- Penberthy KK, Ravichandran KS. 2016. Apoptotic cell recognition receptors and scavenger receptors. *Immunol Rev.* (269):44–59.
- Rhoades KR, Heddleston KL, Rebers PA. 1967. Experimental hemorrhagic septicemia: Gross and microscopic lesions resulting from acute infections and from endotoxin administration. *Can J Comp Med Vet Sci.* (31):226–233.
- Sabiel YA, Musa MT, Zyl AV. 2012. Identification of *Mannheimia haemolytica* and *Pasteurella multocida* by Polymerase Chain Reaction and Random Amplification of Polymorphic DNA. *Sud. J. Vet. Res.* (27): 55–59.
- Sarangi LN, Priyadarshini A, Kumar S, Thomas P, Gupta SK, Nagaleekar VK, Singh VP. 2014. Virulence Genotyping of *Pasteurella multocida* Isolated from Multiple Hosts from India. *Sci. World J.* 2014.
- Shivachandra SB, Viswas KN, Kumar AA. 2011. A review of hemorrhagic septicemia in cattle and buffalo. *Animal Health Research Reviews.* 12(1): 67–82.
- Singh K, Ritchey JW, Confer AW. 2011. *Mannheimia haemolytica*: Bacterial-Host interactions in bovine pneumonia. *Vet. Pathol.* (48): 338–348.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* (30): 2725–2729.
- Tang X, Zhao Z, Hu J, Wu B, Cai X, He Q, Chen H. 2009. Isolation, antimicrobial resistance and virulence genes of *Pasteurella multocida* strains from swine in China. *J. Clin. Microbiol.* (47): 951–958.
- Townsend KM, Hanh TX, Boyle DO, Wilkie I, Phan TT, Wijewardana TG, Trung NT, Frost AJ. 2000. PCR detection and analysis of *Pasteurella multocida* from the tonsils of slaughtered pigs in Vietnam. *Vet Microbiol.*(72): 69-78.
- Verma R, Jaiswal TN. 1998. Haemorrhagic septicemia vaccines. *Vaccine.* 16(11-12): 1184–1192.