Investigation of iron uptake and virulence gene factors (fur, tonB, exbD, exbB, hgbA, hgbB1, hgbB2 and tbpA) among isolates of Pasteurella multocida from Iran

Motahare Feizabadi Farahani¹, Majid Esmaelizad¹*, Ahmad Reza Jabbari²

¹Department of Central Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization, Tehran, Iran
²Department of Pasteurella National Research Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization, Tehran, Iran

Received: July 2018, Accepted: February 2019

ABSTRACT

Background and Objectives: Iron is an essential compound in metabolic pathway of wide range of organisms. Because of limited free iron supply in mammalian and avian hosts, bacteria have applied various ways to acquire iron.

Materials and Methods: In this study, the frequency of 8 iron acquisition factors was examined among 63 avian and ovine Pasteurella multocida field isolates and their vaccine strains using PCR method.

Results: Five candidate genes (fur, tonB, exbD, exbB and hgbA) were identified among all isolates. For the first time, 2 loci (hgbB1 and hgbB2) of the hgbB gene were identified, which were previously reported as 1 gene. Also, it was found that 5 ovine and 1 avian isolates possessed all the virulence factors, which could also be considered for evaluating the frequency of other virulence factors.

Conclusion: More studies need to be conducted on the frequency of all other virulence factors among these isolates, which can provide basic information for improvement or substitution of current vaccinal strains. Overall, as the new designed sets of primers showed more potential in detecting the corresponded genes, researchers can consider them in further studies.

Keywords: Pasteurella multocida; Virulence factors; Iron acquisition

INTRODUCTION

Pasteurella multocida (P. multocida) is a Gram-negative bacterium that belongs to the Pasteurellaceae family (1, 2). It is a nonmotile, facultative anaerobic microbe which can be isolated from a variety of species, including domesticated mammals and poultry throughout the world (3, 4). P. multocida
causes atrophic rhinitis in swine, snuffle in rabbits, fowl cholera among avian, haemorrhagic septicaemia in both bovine and ovine herds (2, 5). There are 5 capsular serogroups: A, B, D, E and F; and according to lipopolysaccharide antigen expression, they are arranged into 16 somatic serotypes (6).

Iron is a crucial compound for most organisms, due to its essential function in metabolic transport chains (3). Bacteria apply various systems to acquire iron such as siderophores or outer membrane proteins (7). Siderophores are iron ligands which compete with protein carriers to bind to ferric iron, while some outer membrane proteins can also acquire iron from the host Fe-binding molecules, such as lactoferrin, heme, ferritin, hemoglobin, and transferrin (4, 8).

Various virulence factors (VF) found in P. multocida include proteins with functions associated with adherence and colonization (PtfA, FimA, Hsf-1, Hsf-2, PhfA and TadD), iron acquisition proteins (ExbB, ExbD, TonB, HgbA, HgbB and Fur), extracellular enzymes such as neuraminidases (NanB and NanH) and superoxide dismutases (Soda, SodC and TbpA), hyaluronidase (PmHAS), toxins (ToxA), lipopolysaccharides (LPS), capsular and outer membrane proteins (OmpA, OmpH, Oma87 and PlpB) (9, 10).

Numerous crucial functions have been reported about the correlation between adhesion proteins and bacterial virulence, provoking adherence and colonization. Sialic acid can be found in a conjugated form to glycolipids and glycoproteins of eukaryotic cells. The presence of sialidases enzymes is crucial in removing these compounds. Additionally, OmpH and PlpE are protective antigens which have been detected in A:1, A:3, and A:4 serotypes from poultry suffered from fowl cholera and cattle with shipping fever (10).

Gram-negative bacteria can apply 2 different types of hemoglobin receptor proteins to boost the transmission of either iron or heme group into the cell. Some extract and deliver the heme group into the cell from hemoglobin by secretating a hemoglobin-binding protein while the other use a specific receptor which can interact with haemoglobin directly and facilitate the transmission of both iron and heme group. Overall, transmission of either iron or heme across the outer membrane by all of the mentioned mechanisms requires the expression of the tonB, exbD and exbB genes (7).

TonB induces the transfer of energy required for transportation of iron into bacterial cells by linking both cytoplasmic and outer membranes of the bacteria. Presence of ExbD and ExbB proteins, as the inner membrane stabilizer for TonB protein, is essential for this process (11).

Regulation of gene expression associated with iron is generally controlled by fur gene in Gram-negative bacteria (12). When iron is abundant, fur interacts with ferrous iron and binds to the conserved promoter regions known as Fur box and inhibits gene transcription (13).

HgbA and HgbB are 2 proteins that are used by P. multocida to acquire iron directly from the haem component. HgbA gene is reported to be distributed more regularly among isolates, whereas hgbB gene prevalence varies among strains according to the host origin and the animal disease status. Another protein known as TbpA, a transferrin binding protein, is reported as an epidemiological marker among cattle and plays an essential role in extraction of iron from transferrin (2).

Few studies have investigated the association between various hosts and frequency of the mentioned VFs among P. multocida isolates which can significantly increase the current knowledge about its pathogenicity, epidemiology, and development of current vaccinal strains (14, 10). Thus, findings of this study can shed light on correlation between the host and frequency of various iron uptake VFs among Iranian P. multocida isolates from different regions and the efficiency of current avian and ovine vaccinal strains on recent field isolates.

MATERIALS AND METHODS

Bacterial samples. A total of 63 lyophilized P. multocida isolates (30 avian and 33 ovine isolates, available in Pasteurella National Research Laboratory at Razi Vaccine and Serum Research Institute) from different provinces of Iran, were used to investigate the presence and prevalence of 8 iron virulence factor genes (fur, tonB, exbD, exbB, hgbA, hgbB1, hgbB2, tbpA). The frequency of hgbB amplicon was also investigated among the isolates. The information related to origin, host, and serotype of each avian isolates are shown in Table 1, but the related information about the ovine isolates was not available.

DNA extraction and concentration. Genomic
DNA was extracted via boiling method from a 24-hour blood agar culture. The process included 1mL of each fresh blood agar culture dissolved in 1 mL of 1x TE buffer made up of 10mM of Tris-HCl (PH 8) and 1mM of EDTA; then, they were put in boiled water (water bath) for 15 minutes. The samples were centrifuged for 5 minutes at 10000 xg. Supernatant, which contained DNA, was used for PCR tests (15).

The quality and quantity of the extracted DNA was evaluated at the OD of 260-280 nm using a spectrophotometer (Eppendorf, Germany) (14).

Conventional PCR program. The primers’ sequences and the amplicon size of PCR products are shown in Table 2.

The PCR program for every primer pair is shown in Table 3. All reactions were done in 20 μL of the total volume. For all reactions, 1 μL of DNA template was added to the 10 μL Master Mix (Ampliqonco), with 7 μL of double-distilled water, and 1 μL from 10 pmol of each primer pair. Specifc P. multocida PCR (kmt1 gene) and isolates’ capsular type were investigated using approved primer sets and PCR protocols (16).

Primer designing. All new sets of primers were designed based on all existed sequences in GeneBank. The first set of primers for detecting fur gene (fur1), used by previous researchers, were aligned in NCBI site (6, 7). Primer sequence was investigated among all the samples that belonged to P. multocida. In forward primer sequence, the 16th nucleotide, which was reported as G, was found as A nucleotide among the reported nucleotide sequences; and in reverse primer sequence, the last 3’ nucleotide, C, was reported as A nucleotide. These SNPs decreased the covering of target sequences and could increase false negative results.

In this study, 2 specific sets of primers were designed that could specifically detect hgbB gene, with the desired amplicon size of 441 bp.

Electrophoresis. The electrophoresis of PCR products was performed in 1% agarose gel and stained with ethidium bromide. The frequency of each gene is reported in Table 4. The approved PCR products, which confirmed the efficiency of new sets of primers, are shown in Table 5.

Data analysis. Matrix 0-1 was designed based on the presence or absence of 8 iron virulence factor genes (fur, tonB, exbD, exbB, hgbA, hgbB1, hgbB2, iltPA).

Data were analysed by DendroUPGMA software (2002) and are shown in Tables 5 and 6.

RESULTS

Capsular typing output. The specific PCR test based on kmt1 gene was positive for all 63 samples and demonstrated that 30 avian and 33 ovine P. multocida isolates belonged to capsular type A.

Conventional PCR results. Based on the new
designed primer pair in this study, the fur gene was present in all the studied isolates. The designed primers (hgbBl and hgbB2) could amplify these loci in P. multocida full genomes. Among the studied collection, the hgbB gene was present in 100% of avian isolates, while 75% of ovine isolates showed the presence of hgbB gene. The results indicated that 36.6% of avian and

Table 2. Iron acquisition virulence factors, their function, primer sequence, amplicon size, and the source of each primer pair

| Primers       | Description                  | Primer sequence (5'-3')                        | Amplicon Size (bp) | Reference |
|---------------|------------------------------|------------------------------------------------|--------------------|-----------|
| (KMT1) pm     | Identification all           | ATCCGCTATTTACCCAGTGG 460                       | 11                 |
| CAPA          | Capsule protein A            | TGCCAAATCGCAGTCAG 1044                        | 11                 |
| CAPB          | Capsule protein B            | CATTATCCAAGCTCCACC 758                         | 11                 |
| Fur1          | Ferric uptake regulation     | GTTTACCGTGTTAGACCA 244                         | 7                  |
| Fur2          | Ferric uptake regulation     | AAAGCGGGGYTGAAAATACG 390                       | This study         |
| TonB          | Iron acquisition             | CGACCGGTGAAACCTAGCCA 261                       | 7                  |
| TonB-exbBD    | Iron acquisition             | GGTGTTGATATGAGCGGC 1144                       | 5                  |
| HgbA          | Hemoglobin-binding protein   | TGGCGGATATGGACTCAAG 419                       | 5                  |
| HgbB          | Hemoglobin-binding protein   | ACCCGGTGGGAATTATGATTG 788                      | 5                  |
| HgbB1         | Hemoglobin-binding protein   | CGTTTCTACTTTTGTCTTGAA 441                      | This study         |
| HgbB2         | Hemoglobin-binding protein   | CGTTTCTACCTTGGTGTGGATG 441                     | This study         |
| TbpA          | Transferrin-binding protein  | ACAACGTTCTGCTCCAG 899                         | This study         |

Table 3. PCR conditions for Iron virulence factors

| Primers       | Initial denaturation (°C / sec) | Denaturation (°C/sec) | Annealing (°C/sec) | Extension (°C/sec) | Final extension (°C/ min) | No. of complete cycles |
|---------------|--------------------------------|-----------------------|--------------------|--------------------|--------------------------|-------------------------|
| KMT1          | 94°C, 3 min                    | 94°C, 1 min           | 51°C, 30 s         | 72°C, 45 s         | 72°C, 5 min              | 35                      |
| CAPA, CAPB    | 93°C, 3 min                    | 93°C, 1 min           | 51°C, 30 s         | 72°C, 45 s         | 72°C, 5 min              | 35                      |
| Fur1          | 93°C, 3 min                    | 93°C, 1 min           | 51°C, 20 s         | 72°C, 20 s         | 72°C, 5 min              | 35                      |
| Fur2          | 93°C, 3 min                    | 93°C, 1 min           | 52°C, 30 s         | 72°C, 40 s         | 72°C, 5 min              | 35                      |
| TonB          | 93°C, 3 min                    | 93°C, 1 min           | 54°C, 30 s         | 72°C, 30 s         | 72°C, 5 min              | 35                      |
| TonB-exbBD    | 93°C, 3 min                    | 93°C, 1 min           | 52°C, 30 s         | 72°C, 1 min        | 72°C, 5 min              | 35                      |
| HgbB          | 93°C, 3 min                    | 93°C, 1 min           | 52°C, 30 s         | 72°C, 40 s         | 72°C, 5 min              | 35-37                   |
| HgbA, HgbB1, HgbB2 | 93°C, 3 min               | 93°C, 1 min           | 52°C, 30 s         | 72°C, 40 s         | 72°C, 5 min              | 35-38                   |
| TbpA          | 93°C, 3 min                    | 93°C, 1 min           | 52°C, 30 s         | 72°C, 1 min        | 72°C, 5 min              | 35-38                   |
54.54% of ovine isolates possessed locus 1. Additionally, locus 2 was present in 80% of avian and 27.27% of ovine isolates. Both loci were present in 20% of avian and 15.15% of ovine isolates. Also, 16.66% of poultry and 39.39% of ovine isolates contained locus 1, while locus 2 was present in 60% and 12.12% of avian and ovine isolates, respectively.

With the exception of tbpA, the fur, hgbA, exbB, exbD and tonB genes were present in all avian and ovine isolates. Moreover, tbpA was detected in all ovine isolates, whereas only 23.3% of avian isolates possessed this gene (Table 4).

**Confirmation of new primer pairs potential.** The PCR products of new sets of primers were sequenced and aligned with all present sequences of *P. multocida* in GeneBank. The fur gene has been submitted with KX832975.1, KX832974.1 accession numbers and KX781178.1, KX781177.1, KX781176.1 are related to tbpA gene.

**Dendrogram analysis.** Based on the presence of fur2, tonB, exbB, exbD, hgbA, tbpA gene, and hgbB1 and hgbB2 loci, the avian and ovine isolates were clustered into 7 and 4 groups, respectively. Moreover, 15.15% of ovine and 3.3% of avian isolates possessed all the VFs in this study. The determinant factors in grouping the avian and ovine isolates concerning the iron uptake VFs were *tbpA*, *hgbB1* and *hgbB2* loci, while all other VFs were present in all isolates. Eventually, avian vaccine strain covered 43.3% of all avian isolates, while the bovine vaccine strain could only cover 33.33% of all ovine field isolates (Tables 5, 6).

### Table 4. Frequency of the evaluated iron uptake VFs in this study among Iranian avian and ovine isolates

| Reference | Host   | Genes  | Capsular Origin |
|-----------|--------|--------|-----------------|
|           |        | *fur1* | *fur2* | *hgbA* | *hgbB* | *hgbB1* | *hgbB2* | *exbD* | *exbB* | *exbBD-TonB* | *tbpA* |          |
| This study| Avian  | 93.3   | 100    | 100    | 100    | 36.66   | 80      | 100    | 100    | 100    | 23.3 | A Iran |
| This study| Ovine  | 93.74  | 100    | 100    | 75     | 54.54   | 27.27   | 100    | 100    | 100    | 100  | A Iran |

### Table 5. Classification of 30 isolates of *P. multocida* from poultry based on the prevalence of iron virulence factors

| Group | Absent genes | Isolate name | Number | Percentage |
|-------|--------------|--------------|--------|------------|
| I     | HgbB2-       | PM30         | 1      | 3/3%       |
| II    | HgbB2-, TbpA-| PM28, PM26, PM22, PM06 | 4     | 13/3%     |
| III   | HgbB1-       | PM29, PM20, PM17, PM08 | 5     | 16/6%     |
| IV    | TbpA-        | PM25, PM23, PM15, PM07, PM03 | 5     | 16/6%     |
| V     | All Positive | PM04         | 1      | 3/3%       |
| VI    | HgbB1-, TbpA-| PM27, PM24, PM19, PM18, PM16, PM14, PM13, PM12, PM11, PM09, PM05, PM02, PM01 | 13    | 43/3%     |
| VII   | HgbB1-, HgbB2-, TbpA- | PM10 | 1     | 3/3%       |

### Table 6. Classification of 33 ovine *P. multocida* isolates according to the prevalence of iron virulence factors investigated in this study

| Group | Absent genes | Isolate name | Number | Percentage |
|-------|--------------|--------------|--------|------------|
| I     | All positive | PM30, PM27, PM22, PM06, PM08 | 5      | 15.15     |
| II    | hgbB2-       | PM32, PM31, PM29, PM28, PM26, PM17, PM16, PM15, PM12, PM11, PM05, PM07 | 12    | 36.36     |
| III   | hgbB1-       | PM23, PM21, PM18, PM09 | 4      | 12.12     |
| IV    | hgbB1-, hgbB2-| PM25, PM24, PM20, PMb2, PM19, PM14, PM13, PM04, PM03, PM02, PM01 | 11    | 33.33     |
DISCUSSION

Epidemiological investigations on frequency of bacterial pathogens can help discover the origin of related diseases, which can further provide essential information for applying more efficient control measures (2).

Only few studies have previously investigated the prevalence of hgbB gene among P. multocida isolates. The primer pair, which was used by most researchers, could amplify both loci of this gene in P. multocida full genomes (hgbB). For the first time, in this study, the prevalence of each copy among avian and ovine isolates was examined using specific sets of primers (hgbB1 for locus1 and hgbB2 for locus 2). The hgbB gene was present in 100% of avian isolates, while 75% of ovine isolates showed the presence of this gene. Also, the presence of hgbB gene among avian isolates has been reported to be 100% in Iranian and Brazilian, 85% in German, and 76.9% in Indian isolates. Moreover, it has been demonstrated that 100% of Iranian and Indian cattle isolates possessed this gene, while some other studies indicated the presence of this gene in 57.4, 57.7, and 61.3% of ovine and cattle isolates from Iran, Germany, and Japan, respectively (2, 5, 9, 17-20). In contrast, it has been reported that only 40.9% of Iranian cattle and buffalo isolates from Khuzestan possessed this gene (10).

HgbA was present in 100% of ovine and avian isolates investigated in this study. The frequency of this gene among different hosts, irrespective of their origin, has been reported from 84.6% to 100%, while it was present in 73.9% of Brazilian rabbit isolates (2, 5, 6, 7, 17, 19, 20). In addition, a previous study on Iranian cattle and buffalo isolates indicated the presence of hgbA gene among 77.2% of all studied isolates (10).

The presence of fur gene in 93.3% of avian and 93.74% of ovine isolates was detected by previous published primers (Fur1). Sequence alignment of fur gene observed 2 SNPs in fur1 primer positions. In this study, 2 primer pairs were used to investigate the frequency of fur gene among isolates. Using a new primer set (fur2), the positive results were increased to 100% in comparison to the previously published primer (Fur1). Meanwhile, fur1 primer covered 82.6% of Iranian cattle isolates (9). Additionally, 96.7% of swine isolates that belonged to China possessed this gene (6). Ferreira (2012) found that the frequency of fur gene was only 4.3% among Brazilian rabbit isolates (20). Also, tonB, exbB and exbD genes are transcribed independently while are physically linked. The iron carrier components (transferrin, siderophore, haemoglobin) possess a conserved region known as tonB box, which is responsible for interaction with TonB protein. Binding of TonB box to the outer membrane receptor induces a change in TonB protein conformation and passes iron through TonB pore (11). The expression level of tonB, exbD and exbB alternation in response to low iron condition has been reported to be 2.5, 2.3 and 4.7 folds (3).

Furthermore, tonB, exbB and exbD were present among all ovine and avian isolates investigated in this study. Previous research showed the presence of tonB gene to be between 94.6% and 100% among P. multocida isolates from different regions and hosts. Also, exbD and exbB genes were frequency reported to be between 98.9%-100% and 82%100%, respectively (5, 6, 9, 19). Also, tonB-exbBD primer set could amplify all 3 tonB, exbB and exbD genes, which has been detected among 60.8% of Brazilian rabbit isolates (20). Results of this study demonstrated that the frequency of tonB-exbBD was 100% among studied isolates. Previous research indicated that exbBD-tonB amplicon was also present in a high rate (90.9%) among buffalo and cattle isolates from Khuzestan province in Iran (10). TbPA is an iron-binding protein, which interacts directly with host iron-loaded glycoproteins and is expressed on the outer membrane of the bacterial cell (21). In this study, tbPA gene was present in 23.3% of poultry and 100% of ovine isolates. Previous researches reported the presence of this gene in 69.2% of poultry isolates from India (2). However, none of the avian isolates from Germany possessed this gene (5). The presence of this gene among cattle and sheep isolates was reported to be between 69%-100% in different regions. Also, 8.6% of rabbit isolates from Brazil contained this gene (18-20).

Moreover, new primer pairs which were designed in this study, for fur, 2 loci of hgbB gene (hgbB1, hgbB2) and tbPA genes, indicated more efficiency in detecting these genes among Iranian avian and ovine isolates. It is suggested that these primers can be used to ensure a precise detection of the target genes in further studies. Moreover, not any specific relationship between the frequency of investigated VFs and host and/or geography was observed among avian isolates. It has been stated that a suitable vaccine strain should contain the most virulence and immunogenic
factors. In fact, the results of this study indicated that the indicated avian and bovine strains could not cover all the iron uptake VFs, while there are some avian (PM04) and ovine (PM30, PM27, PM22, PM06, and PM08) field isolates which demonstrated 100% coverage. The comparison between the vaccine strains and field isolates need to be investigated and updated continuously. Therefore, the results of this study, in addition to evaluating the presence of other VFs, can provide basic information for substituting current vaccines or choosing suitable isolates for polyvalent or recombinant vaccine strains in future.

**ACKNOWLEDGEMENTS**

Authors are grateful to Razi Vaccine and Serum Research Institute for providing financial support for this project (Grant number:18-18-94104).

**REFERENCES**

1. Jabbari AR, Esmailizad M, MoazeniJula GhR. Polymerase chain reaction typing of *Pasteurella multocida* capsules isolated in Iran. Iran J Vet Res 2006; 7:50-55.
2. Sarangi LN, Priyadarshini A, Kumar S, Thomas P, Gupta SK, Nagaleekar VK, et al. Virulence genotyping of *Pasteurella multocida* isolates from multiple hosts from India. *ScientificWorldJournal* 2014; 2014:814109.
3. Paustian ML, May BJ, Kapur V. *Pasteurella multocida* gene expression in response to iron limitation. *Infect Immun* 2001; 69:409-4115.
4. Paustian ML, May BL, Cao D, Boley D, Kapur V. Transcriptional response of *Pasteurella multocida* to defined iron sources. *J Bacteriol* 2002; 184: 6714-6720.
5. Ewers C, Lubke-Becker A, Bethe A, Kiessling S, Filter M, Wieler LH. Virulence genotyping of *Pasteurella multocida* strains isolated from different hosts with various disease status. *Vet Microbiol* 2006; 114:304-317.
6. Tang X, Zhao Z, Hu J, Wu B, Cai X, He Q, et al. Isolation, antimicrobial resistance, and virulence genes of *Pasteurella multocida* strains from swine in China. *J Clin Microbiol* 2009; 47:951-958.
7. Bosch M, Garrido ME, Liagostera M, Pérez de Rozas AM, Badiola A, Barbé J. Characterization of the *Pasteurella multocida* hgbA gene encoding a haemoglobin-binding protein. *Infect Immun* 2002; 70: 5955-5964.
8. Bosch M, Tarragó R, Garrido ME, Campoy S, Fernández de Henestrosa AR, Pérez de Rozas AM, et al. Expression of the *Pasteurella multocida* omph gene is negatively regulated by the Fur protein. *FEMS Microbiol Lett* 2001; 203: 35-40.
9. Khamesipour F, Mottar H, Azhdary Mamoreh M. Occurrence of virulence factors and antimicrobial resistance in *Pasteurella multocida* strains isolated from slaughter cattle in Iran. *Front Microbiol* 2014; 5: 536.
10. Gharibi D, Hajikolaei M, Ghurbanpour M, Barzegar K. Virulence gene profiles of *Pasteurella multocida* strains isolated from cattle and buffalo. *Vet Arh* 2017; 87: 677-690.
11. Bosch M, Garrido ME, Liagostera M, Perez de Rozas AM, Badiola I, Barbe J. *Pasteurella multocida* exbB, exbD and tonB genes physically linked but independently transcribed. *FEMS Microbiol Lett* 2002; 210: 201-208.
12. van Vliet AH, Wooldridge KG, Ketley JM. Iron-responsive gene regulation in a campylobacter jejuni fur mutant. *J Bacteriol* 1998; 180: 5291-5298.
13. Mey AR, Wyckoff EE, Kanukuthy V, Fisher CR, Payne SM. Iron and fur regulation in *Vibrio cholera* and the role of fur in virulence. *Infect Immun* 2005; 73:8167-8178.
14. Haghazari S, Jabbari AR, Tadayon K. Prevalence of adhesion virulence factor genes, anti-gram, and pathogenicity of avian *Pasteurella multocida* isolate from Iran. *Arch Razi Inst* 2017; 72: 83-91.
15. Haghazari S, Jabbari AR, Tadayon K. Molecular study of virulence factors of *Pasteurella multocida* isolates from poultry in Iran. *Vet J Iran-garmsar* 2016; 12: 101-112.
16. Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJS. Development of PCR assays for species and type-specific identification of *Pasteurella multocida* isolates. *J Clin Microbiol* 1998; 36:1096-1100.
17. Furian TQ, Borges KA, Rocha SLS, Rodrigues EE, do Nascimento VP, Salle CTP, et al. Detection of virulence-associated genes of *Pasteurella multocida* isolated from cases of fowl cholera by multiplex-PCR. *Pesq Vet Bras* 2013; 33:177-182.
18. Shayegh J, Atashpaz S, Hejazi MS. Virulence gene profile and typing of *Pasteurella multocida*. *Asian J Anim Vet Adv* 2008; 3: 206-213.
19. Katsuda K, Hoshino K, Ueno Y, Kohimoto M, Kimaki O. Virulence genes and antimicrobial susceptibility in *Pasteurella multocida* isolates from calves. *Vet Microbiol* 2013; 167:737-741.
20. Ferreira TS, Felizardo MR, Sena de Gobbi DD, Gomes CR, Nogueira Filsner PH, Moreno M, et al. Virulence genes and antimicrobial resistance profiles of *Pasteurella multocida* strains isolated from rabbits in Brazil. *ScientificWorldJournal* 2012; 2012: 685028.
21. Atashpaz S, Shayegh J, Hejazi MS. Rapid virulence typing of *Pasteurella multocida* by multiplex PCR. *Res Vet Sci* 2009; 87: 355-357.