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RISK FACTORS AND PCR STUDIES ON *PASTEURELLA MULTOCIDA* AND *MANNHEIMIA HAEMOLYTICA ISOLATED* FROM PNEUMONIC CATTLE AND BUFFALOES IN EGYPT

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ABSTRACT

Pasteurellosis is one of the important economic diseases in ruminants; especially in cattle and buffaloes. It is caused by *Pasteurella multocida* and *Mannheimia haemolytica*. In the current study, a total number of 128 nasal swabs were collected at winter and summer seasons from pneumonic cattle and buffaloes at different age groups from different localities. Nasal swabs were bacteriologically examined. Three *P. multocida* isolates and six *M. haemolytica* isolates were recovered from 67 pneumonic cattle with isolation rate 4.5% and 8.9 %, respectively. Also, three *P. multocida* isolates and six *M. haemolytica* isolates were recovered from 61 pneumonic buffaloes with isolation rate 4.9% and 9.8%, respectively. The isolation rate was correlated to some epidemiological (risk) factors such as season, age and sex. In case of *P. multocida* the highest isolation rate was in animals belonging to (6-12 month) followed by animals belongs to (12-24 month) age groups followed by (0 up to 6 month) age group while there is no isolation from animals belonging to (>24 month) age group; the highest seasonal rate was in winter season and it was recorded in males. In case of *M. haemolytica*, the highest isolation rate was in animals belonging to (6-12 month) followed by (12-24 month) followed by (0 up to 6 month) age groups while no isolation was recorded from (>24 month) age group; the highest isolation rate was in summer season and it was observed in males. All recovered isolates were biochemically characterized and confirmed by amplification of *kmt1* gene for *P. multocida* isolates and *rpt* gene for *M. haemolytica* isolates using PCR technique.

Key words:

Pneumonia, cattle, buffaloes, *P. multocida*, M. *haemolytica*, *kmt1*and *rpt*.

INTRODUCTION

Bovine respiratory disease (BRD) is among the most important diseases of the cattle industry worldwide, causing great economic losses to farmers and animal owners by reducing average daily gain, feed efficiency, overall performance of beef calves and mortality **(Taylor *et al.* 2010; Hartel *et al.* 2004)**. Pneumonic pasteurellosis refers to any of the disease conditions caused by bacteria of the genera *Pasteurella or Mannheimia* **(Adamu and Ameh, 2007)**. The typical clinical disease is highly infectious, often fatal and with very serious economic impact in animal industry. It is well established that pneumonic pasteurellosis is responsible for the largest cause of mortality in feedlot animals in which the disease accounts for approximately 30% of the total cattle deaths worldwide **(Mohamed and Abdulsalem, 2008)**. It is worth mentioned that *M. haemolytica* and *P. multocida* constitute the most important members of the family *Pasteurellaceae* that pose serious hazards in livestock industry **(Babetsa *et al.* 2012)**. The complexities associated with conventional diagnostic methods for *P. multocida* and *M. haemolytica* can be overcome by PCR **(Townsend *et al.* 1998; Jaramillo-Arango *et al.* 2007)**. The present study was undertaken to determine the frequency of isolation of *P. multocida* and *M. haemolytica* from pneumonic cattle and buffaloes.

MATERIAL AND METHODS

Animals:

A total number of 67 cattle and 61 buffaloes showing clinical signs of respiratory affection of different ages of both sexes in two seasons (winter and summer) were examined during the period from October 2015 till June 2016. These animals were belonging to different farms located in EL-Fayoum, EL-Giza, EL-Gharbiya, AL-Menofiya and EL-Behera Governorates.

Samples:

Nasal swabs were collected from 67 cattle and 61 buffaloes suffering from respiratory manifestations using sterile cotton swabs and transferred to the laboratory on ice box for bacteriological examination.

Microbiology culturing and biochemical tests:

Nasal swabs were inoculated into brain heart infusion broth and incubated at 37^{o}c for 6-8 hrs. for propagation of micro-organisms then a loopful from brain heart infusion broth was streaked on sheep blood agar and MacConkey agar plates and incubated at 37°C for 24 hrs. for primary differentiation of the pathogen following standard procedures. Colonies were

characterized and those giving Gram-negative coccobacilli or short rods with or without bipolar staining on smears were subcultured for identification. A 24 h pure suspected culture (isolate) was subjected to biochemical tests using standard procedures according to **(Quinn *et al.* 2002).**

Mice pathogenicity test:

The suspected *P. multocida* isolates were inoculated in brain heart infusion broth and incubated at 37°C for 6-8 hours and then 0.5 ml of bacterial cultures were inoculated intraperitoneally into mice which kept under observation for 72 hours after inoculation. Dead mice were inspected for *P. multocida* lesions and blood films were prepared from heart blood and stained with Leishman's stain for detection of bipolarity **(Carter, 1967).**

After complete identification, the bacterial isolates were stored at - 20°C in brain heart infusion broth containing 16% glycerol for long term preservation.

Identification of *P. multocida* and *M. haemolytica* isolates by Polymerase chain reaction:

DNA extraction: DNA was extracted from bacterial colonies using QIAamp DNA mini kit (Qiagen, Germany, GmbH) instructions.

Oligonucleotide primers: Primer used were supplied from (Metabion Company, Germany) are listed in (Table 1).

PCR amplification:

Primers were utilized in a 25 µl reaction containing 5 µl of 5x Taq PCR Master Mix (Jena Bioscience, Germany),1 µl of each primer of 20 pmol concentration, 13 µl of PCR grade water (Jena Bioscience, Germany) and 5 µl of DNA template. The reaction was performed in a Biometra thermal cycler.

Analysis of the PCR products:

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Bio shop, Canada) in 1X TAE buffer at room temperature. For gel analysis, 6 µl of the products were loaded in each gel well. A 100 bp DNA ladder (Jena Bioscience, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech Biometra) and the data was analyzed through (Gel pro analyzer®) software version 4.

Table (1): Primer sequence, target gene, amplicon size and cycling condition.

Target gene	Primer sequence	Amplified segment (bp)	Primary denatura-tion	Amplification (30 cycle)			Final extension	References
				Secondary denaturation	Annealing	Extension		
KMT1	KMT1T7: ATCCGCTATTTACCCAGTGG KMT1SP6: GCTGTAAACGAACTCGCCAC	460	95oc 5 min.	95oc 30 sec.	55oc 30 sec.	72oc 30 sec.	72oc 5 min.	Townsend *et al.* (2001)
Rpt	Rpt2: GTTTGTAAGATATCCCATTT Rpt2rev: CGTTTTCCACTTGCGTGA	1022	95°c 3 min.	95°c 1 min.	48°c 1 min.	72°c 30 sec.	72°c 10 min.	Deressa *et al.* (2010)

RESULTS

A total number of 3 *P. multocida* isolates and 6 *M. haemolytica* isolates were recovered from 67 pneumonic cattle with isolation rate 4.5% and 8.9%, respectively. Also, three *P. multocida* isolates and 6 *M. haemolytica* isolates were recovered from 61 pneumonic buffaloes with isolation rate 4.9% and 9.8%, respectively. On Blood agar, all recovered *P. multocida* isolates appeared as moderate size, round, greyish mucoid colonies and non-heamolytic while *M. haemolytica* was β-heamolytic. On MacConkey agar, *M. haemolytica* grow as pinpoint red colonies while *P. multocida* didn't grow. They appeared as small, Gram-negative rods or coccobacilli in Gram's stained slides and distinctive bipolar-staining of *P. multocida* seen in Leishman-stained smears. Biochemical identification revealed that, in catalase test *P. multocida* and *M. haemolytica* liberated bubbles of oxygen gas; in oxidase test *P. multocida* and *M. haemolytica* showed dark blue color; on TSI agar *P. multocida* and *M. haemolytica* fermented all sugars and produced yellow slant and butt without H_2S or gas production; on Simmon's citrate agar media *P. multocida* and *M. haemolytica* were unable to utilize citrate and showed greenish color of the media and in indole reaction *P. multocida* produced red ring at the interface while *M. haemolytica* produced yellow ring. All *P. multocida* isolates were highly pathogenic to mice as they cause death of mice within 18-24 hours of inoculation. The results of the amplification of *kmt1*and *rpt* genes using PCR revealed that, all *P. multocida* isolates were positive for *kmt1* gene (100%) showing an

amplicon size (460bp) Fig. (1) and all *M. haemolytica* isolates were positive for *rpt* gene (100%) showing an amplicon size (1022bp) Fig. (2).

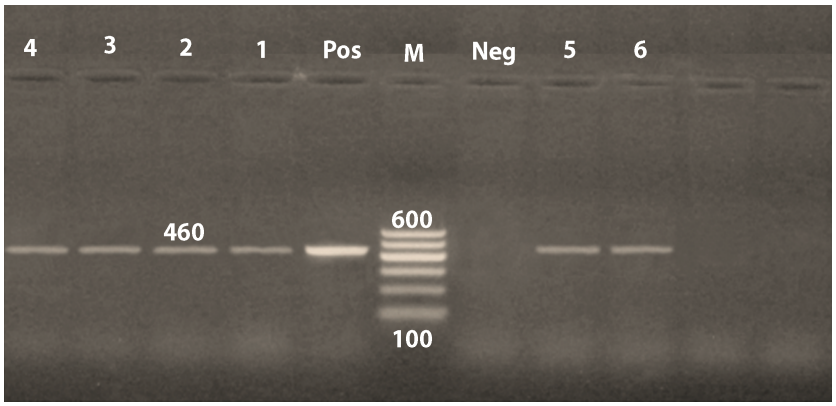

Fig. (1): Agarose gel electrophoresis of PCR for detection of *Kmt1* gene in *Pasteurella multocida* strains.

Lane M: Molecular weight marker, 100 - 600 bp.

Lanes 1-6: Positive samples with band of amplicon size at 460 bp.

Lane Pos: Positive control of *Kmt1* gene with band of amplicon size at 460 bp.

Lane Neg: Negative control.

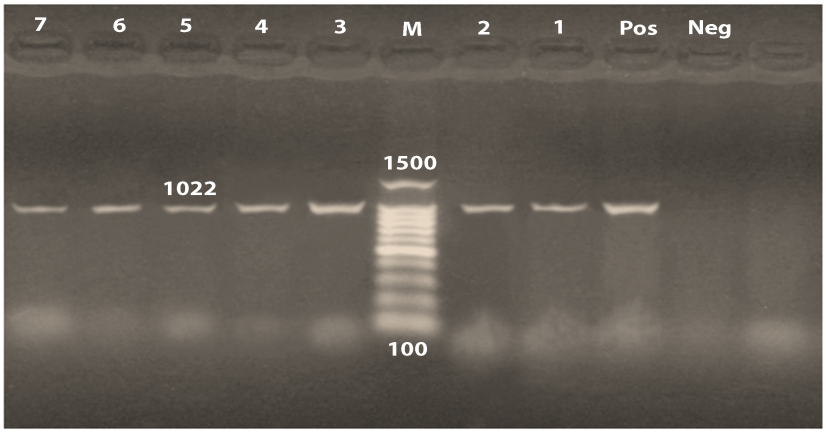

Fig. (2): Agarose gel electrophoresis of PCR for detection of *rpt* gene of *M. haemolytica* strains.

Lane M: Molecular weight marker, 100-1500 bp.

Lanes 1-7: Positive samples with band of amplicon size at 1022 bp.

Lane Pos: Positive control of *rpt* gene with band of amplicon size at 1022 bp.

Lane Neg: Negative control.

The isolation rate was correlated to some epidemiological (risk) factors such as season, age and sex. It was found that in case of *P. multocida,* the highest isolation rate was in animals

belonging to (6-12 month) age group (10.81%) followed by (12-24 month) age group (7.69%) followed by (0 up to 6 month) age group (1.4%) while there is no isolation from (>24 month) age group (Table 2) and Fig. (3); the highest isolation rate was in winter season (4.9%) (Table 4) and Fig.(5) and the highest isolation rate was noticed in males (5.71%) (Table 6) and Fig.(7) while in case of *M. haemolytica*, the highest isolation rate was in animals belonging to (6-12 month) age group (16.21%) followed by (12-24 month) age group (7.7%) followed by (0 up to 6 month) age group (7.04%) while there is no isolation from (>24 month) age group (Table 3) and Fig.(4); the highest isolation rate was in summer season (11.53%) (Table 5) and Fig. (6) and the highest isolation rate was in males (10.48%) (Table 7) and Fig. (8).

Table (2): Isolation rate of *P. multocida* from clinically diseased cattle and buffaloes in correlation with age

Age (months)	Total No. of buffaloes	Isolation rate in buffaloes		Total No. of cattle	Isolation rate in cattle		Total No. of isolates	% of total isolates
		No. of positive cases	%		No. of positive cases	%		
Up to 6 (71)	46	1	2.2	25	0	0	1	1.4
6-12 (37)	12	2	16.7	25	2	8	4	10.81
12-24(13)	-	-	-	13	1	7.7	1	7.69
More than 24 (7)	3	0	0	4	0	0	0	0
Total (128)	61	3	4.9	67	3	4.5	6	4.69

Table (3): Isolation rate of *M. haemolytica* from clinically diseased cattle and buffaloes in correlation with age

Age (months)	Total No. of buffaloes	Isolation rate in buffaloes		Total No. of cattle	Isolation rate in cattle		Total No. of isolates	% of total isolates
		No. of positive cases	%		No. of positive cases	%		
Up to 6 (71)	46	5	10.9	25	0	0	5	7.04
6-12 (37)	12	1	8.3	25	5	20	6	16.21
12-24 (13)	-	-	-	13	1	7.7	1	7.7
More than 24 (7)	3	0	0	4	0	0	0	0
Total (128)	61	6	9.84	67	6	8.9	12	9.37

Table (4): Isolation rate of *P. multocida* from clinically diseased cattle and buffaloes in correlation with season.

Season	Total No. of buffaloes	Isolation rate in buffaloes		Total No. of cattle	Isolation rate in cattle		Total No. of isolates	% of total isolates
		No. of positive cases	%		No. of positive cases	%		
Winter (102)	61	3	4.9	41	2	4.87	5	4.9
Summer (26)	-	-	-	26	1	3.8	1	3.84
Total (128)	61	3	4.9	67	3	4.5	6	4.69

Table (5): Isolation rate of *M. haemolytica* from clinically diseased cattle and buffaloes in correlation with season.

Season	Total No. of buffaloes	Isolation rate in buffaloes		Total No. of cattle	Isolation rate in cattle		Total No. of isolates	% of total isolates
		No. of positive cases	%		No. of positive cases	%		
Winter (102)	61	6	9.84	41	3	7.3	9	8.8
Summer (26)	-	-	-	26	3	11.5	3	11.53
Total (128)	61	6	9.84	67	6	8.9	12	9.37

Table (6): Isolation rate of *P. multocida* from clinically diseased cattle and buffaloes in correlation with sex.

Sex	Total No. of buffaloes	Isolation rate in buffaloes		Total No. of cattle	Isolation rate in cattle		Total No. of isolates	% of total isolates
		No. of positive cases	%		No. of positive cases	%		
Male (105)	46	3	6.5	59	3	5.1	6	5.71
Female (23)	15	0	0	8	0	0	0	0
Total (128)	61	3	4.9	67	3	4.5	6	4.69

Table (7): Isolation rate of *M. haemolytica* from clinically diseased cattle and buffaloes in correlation with sex.

Sex	Total No. of buffaloes	Isolation rate in buffaloes		Total No. of cattle	Isolation rate in cattle		Total No. of isolates	% of total isolates
		No. of positive cases	%		No. of positive cases	%		
Male (105)	46	6	13	59	5	8.5	11	10.48
Female (23)	15	0	0	8	1	12.5	1	4.35
Total (128)	61	6	9.84	67	6	9	12	9.37

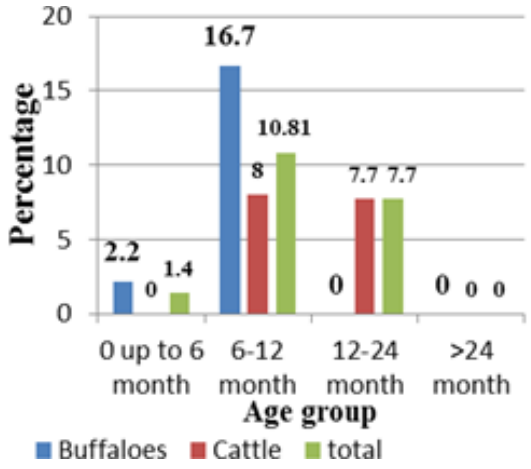

Fig. (3): Isolation rate of *P.muitocida* from clinically diseased cattle and buffaloes in correlatin. with age.

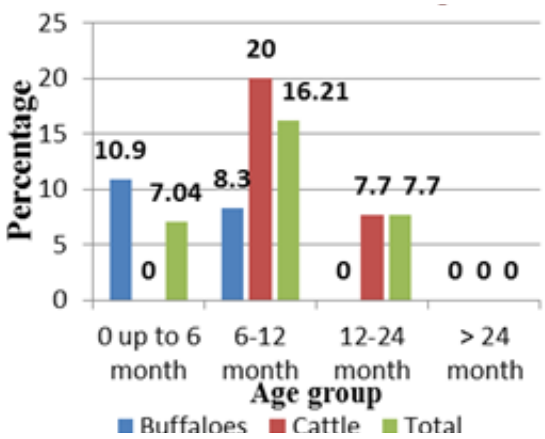

Fig. (4): Isolation rate of *M.haemolytica* from clinically diseased cattle and buffaloes in correlation with age

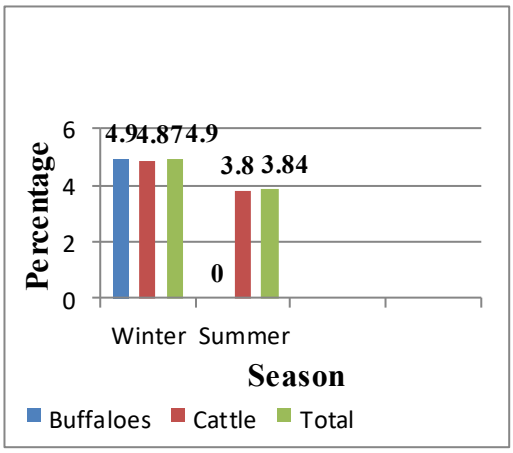

Fig. (5): Isolation rate of *P.multocida* from clinically diseased cattle and buffaloes in correlation with season

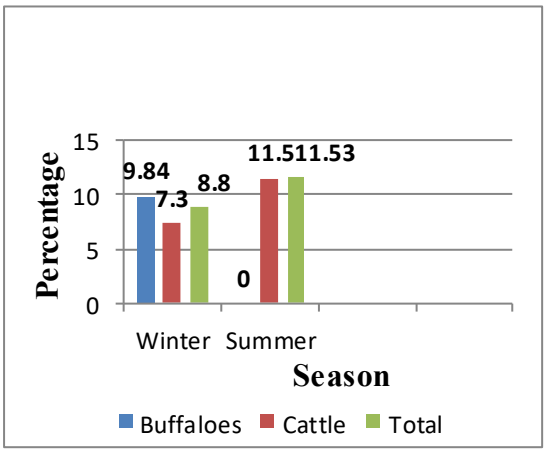

Fig. (6): Isolation rate of *M.haemolytica* from clinically diseased cattle and buffaloes in correlation with season

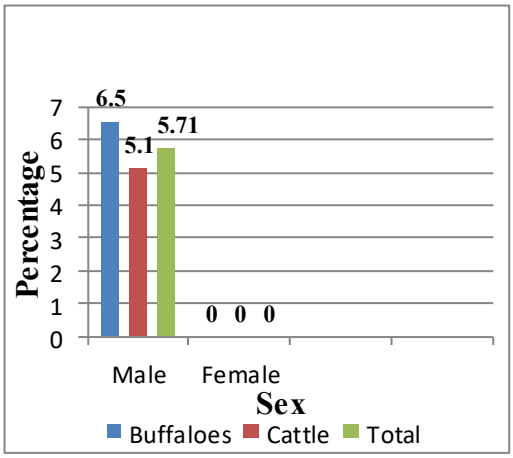

Fig. (7): Isolation rate of *P.multocida* from clinically diseased cattle and buffaloes in correlation with sex

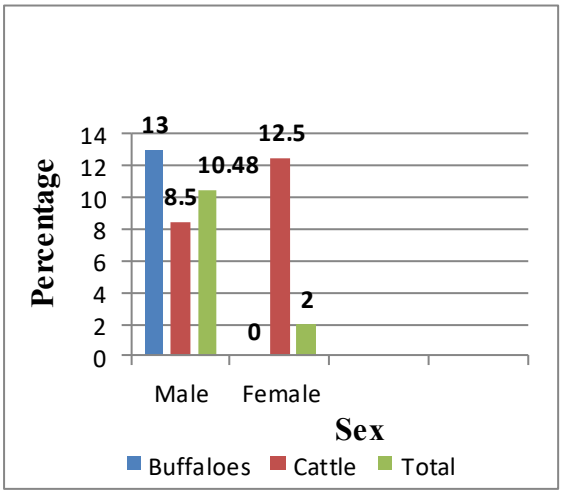

Fig. (8): Isolation rate of *M.haemolytica* from clinically diseased cattle and buffaloes in correlation with sex

DISSCUSION

Pasteurellosis has been considered as a substantial issue in the livestock industry and it is one of the important economic diseases in ruminants, especially in cattle and buffaloes as mentioned by **Sugun *et al.* (2013)**. *Pasteurella multocida* and *Mannheimia haemolytica* are the main causative agents of pneumonic pasteurellosis in cattle as reported by **Kaoud *et al.* (2010) and Karimkhani *et al.* (2011)**. This investigation aimed to determine the rate of *P. multocida* and *M. haemolytica* infection among the examined diseased cattle and buffaloes; correlate the rate of infection among the examined animals with some risk factors such as

species, age, season and sex. In the present study, the total numbers of isolates were 18 (12 *M. haemolytica* in a percentage of 66.66% and 6 *P. multocida* in a percentage of 33.33%) isolates out of 128 examined samples with an isolation rate 14.06%. These findings were higher than that mentioned by **Abera *et al.* (2014)** who found the overall percentage 8.51% with *M. haemolytica* (46.4%) and *P. multocida* (39.3%). Comparing the two *Pasteurella* species, *M. haemolytica* (66.66%) was the major causative agent involved in bovine pneumonic pasteurellosis. This was consistent with **Abera *et al.* (2014)**. On the other hand, **Zaki *et al.* (2002)** detected higher prevalence rate of *P. multocida* (19.9%) in comparison with *M. haemolytica* (8.8%). *M. haemolytica* has been known to be the main bacterial agent responsible for the lung infection and has been known to be higher in acute pneumonia as found by **Daniel *et al.* (2006)** who isolated *M. haemolytica* in a higher percentage than *P. multocida* from severe lung lesions. Regarding the age group susceptibility this study revealed that, the highest rate of infection was in (6 - 12 month) age group followed by (12-24 month) age group followed by (0 up to 6 month) age group while the age group more than 24 month didn't show any isolates and this is nearly similar to the finding of **Abera *et al.* (2014)** and **Aditi *et al.* (2014)** who found higher rate of infection was associated with young age groups as compared to adults. This might be due to the immune status of the animal being able to predispose to the bacterial infection and other predisposing etiological agents. Seasonal variation revealed that, the highest rate of *P. multocida* infection was recorded in winter season (4.9%) which is similar to the finding of **Karimkhani *et al.* (2011)** while in *M. haemolytica*, the highest rate was noticed in summer season (11.53%) and this may be due to the number of collected samples in each season. In this study, it was found that, the highest rate of infection was recorded in males which is similar to the finding of **Karimkhani *et al.* (2011)**. In the present study, the low rate of isolation may be due to other incriminated causes such as mycoplasma, viruses or fungi as the etiology of pneumonia is complex and multifactorial which are either non-infectious or microbial determinants including bacteria, viruses and fungi as mentioned by **Garoia *et al*, (1982)**. Failure of bacteriological isolation in many samples may be attributed to the fact that *Proteus* spp. was present in some of the cultures and swarmed over the bacteria on growth media. This study showed that all isolated *P. multocida* caused death of inoculated mice within 24 hours post inoculation and an overwhelming septicemia has been observed. These results revealed the high virulence of such organism as mentioned by **Naz *et al.* (2012)**. The complexities associated with

conventional diagnostic methods for *P. multocida* and *M. haemolytica* can be overcome by PCR. The PCR assay developed by **Townsend *et al.* (1998)** based on KMT1T7 and KMT1SP6 primers has been widely employed worldwide for initial species identification with field isolates irrespective of capsular types. All isolated *P. multocida* produced a positive signal by an amplicon of approximately 460 bp. Also, PCR assay demonstrated that, all *M. haemolytica* isolates carried the *Rpt* gene as mentioned by **Deressa *et al.* (2010).**

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