Int.J.Curr.Microbiol.App.Sci (2018) *7*(9): 1990-1998

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 7 Number 09 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.709.241

Emergence of Virulent *Pasteurella multocida* and *Mannheimia hemolytica* in Sheep and Goats of Western Maharashtra, India

Sneha H. Daphal, Prashant P. Mhase, Mrunalini M. Pawade*, Prerana P. Shelke, Jyotica D. Sangle and Rahul P. Kolhe

Department of Microbiology, Krantisinh Nana Patil College of Veterinary Science, Shirwal, Satara, Maharashtra, India

**Corresponding author*

Keywords

Pasteurella multocida, Manheimia haemolytica, PCR, CAP A, CAP D, Sheep, PHSSA and LKT gene

Article Info

Accepted:
12 August 2018
Available Online:
10 September 2018

A B S T R A C T

Pasteurella species are often encountered in small ruminants as major pathogens in respiratory illnesses resulting in heavy losses. Present study was aimed towards finding out the occurrence of *Pasteurella* spp. in small ruminants as respiratory pathogen and detection of virulence in them by phenotypic and genotypic characterization. In this study total 208 different samples like nasal swabs, lungs and heart blood were collected from diseased as well as healthy sheep (n=90) and goats (n=118) from the region of Western Maharashtra state of India. All the isolates (n=23) were processed for phenotypic characterization of *Pasteurella* spp. The isolates were confirmed with specific gene polymerase chain reaction by KMT and PHSSA gene PCR and CapA, CapD, and Lkt gene PCR for expression of virulence. Bacteriological examination of samples revealed that, out of 208 samples 23 (11.05%) were found culturally positive for presence of *Pasteurella* spp. Out of total *Pasteurella* spp. isolated, 16/23 (69.56%) were identified as *P. multocida* and 07/23 (30.43%) were identified as *M. haemolytica*. The isolates confirmed by Species specific KMT PCR for *P. multocida* resulted in cent percent positivity (100 %), while 57.12 percent of all phenotypically detected *M. haemolytica* were confirmed by PHSSA PCR. The virulence of *P. multocida* organisms was determined by expression of capsular antigen CapA found in 81.25% isolates and CapD gene found in 50.00 % isolates, while virulence in *M. Haemolytica* was determined by PHSSA and Lkt gene found in 57.12% and 42.85% isolates.

Introduction

Small ruminants plays an important role in the food and nutritional security of millions of rural people especially the landless, marginal and small farmers in arid and semiarid rainfed regions of tropical countries like India. Respiratory illness is observed as a major problem commonly encountered in flocks, affecting groups or individuals of all ages and types of these animals (Naveed *et al.*, 1999). Etiological agents, members of the family *Pasteurella ceae* like *Pasteurella multocida* and *Manheimia haemolytica* are considered as major respiratory pathogensin sheep, goats and cattle (Deressa *et al.*, 2010; Babetsa *et al.*, 2012). Out of five capsular serogroups of *P. multocida* (A, B, D, E, and F), A and D are

usually associated with Pasteurellosis, while serotypes A1 and A2 of *M. haemolytica* are predominant out of twelve (A1, A2, A5-A9, A12-14, A16 and A17) serotypes, where A1 is responsible for pasteurellos is in cattle and A2 for disease in sheep (Angen *et al.,* 1999). Although conventional isolation and phenotypic methods remain a gold standard in proper diagnosis, recently molecular tools have been evolved for quick, precise, sensitive and reliable confirmation and characterization of these organisms.

Polymerase chain reaction (PCR) is a widely accepted, rapid, specific and highly sensitive technique for confirmation of organisms as well as for characterizing the expression of virulence genes. PCR assays are well documented for amplification of specific capsular genes and virulence genes of *P. multocida* and *M. haemolytica* (Townsend *et al.,* 2001; Hawari *et al.,* 2008; Kumar *et al.,* 2015).

Materials and Methods

Phenotypic characterization of isolates

Samples like nasal swabs,lung tissue, lymph node and heart blood were collected aseptically from 208 small ruminants comprising sheep (43 diseased and 47 healthy animals)and 118 goats (61 diseased and 57 healthy animals), respectively. Geographical study area which was selected belonged to three districts Pune, Satara and Sangli of Western Maharashtra, India. The collected samples were immediately transferred in sterile glass vials, labeled and brought over ice to the laboratory, and inoculated in the growth medium for enrichment and isolation of organisms (Ricketts, 1981). The samples were inoculated in brain heart infusion broth (HiMedia, India) and further streaked on 5% sheep blood agar and MacConkey agar. Phenotypic detection of the bacterial species was carried out on the basis of colonial morphology, Gram staining and biochemical characteristics.

Molecular characterization of *Pasteurella* spp.

Isolates of *Pasteurella* spp. which were phenotypically confirmed by cultural and biochemical tests were subjected to PCR for confirmation of species specific gene and expression of virulence gene. Purified DNA of isolates were processed by targeting for presence of species specific KMT gene and capsular antigen gene CAP A and CAP D of *P. multocida* as per method described by Townsend *et al.,* (1998) and Townsend *et al.,* (2001). Similarly isolates of *M. haemolytica* were processed by employing species specific PHSSA gene PCR and expression of virulence by targeting Lkt gene as per method described by Kumar *et al.,* (2015) and Alexander *et al.,* (2008) respectively.

Purification and extraction of bacterial genomic DNA was performed with phenol chloroform method of Sambrook *et al.,* (1989) for template DNA and PCR was performedfor amplification of desired genes employing the published primer sequences (Table 1).

Results and Discussion

On inoculation of samples in brain heart infusion brothit was further streaked on 5% sheep blood agar and MacConkey agar. After incubating for 24 hr at 37˚C isolates of *P. multocida* colonies showed characteristic dew drop, non-haemolytic and translucent colonies on 05% sheep blood agar and no growth was seen on MacConkey. While colonies of *M. haemolytica* showed smooth, grayish, β-haemolytic colonies with pinkish coloured growth on MacConkey agar. On microscopic examination, both types of colonies were Gram negative rods and on Leishman's

staining it showed characteristic bipolar appearance. On biochemical testing, all *P. Multocida* isolates were found positive for indole, catalase, oxidase and negative for methyl red, Voges-Proskaur and citrate utilization tests, respectively. While upon biochemical testing, *M. haemolytica* isolates were found positive to catalase and oxidase while negative to in dole, methyl red, Voges-Proskaur and citrate utilization tests.

Incidence of Pasteurellosis in sheep and goat

All the samples collected from small ruminants were tested culturally for Pasteurellosis and 23/208 (11.05%) were found positive, amongst which 19/104 (18.26%) belonged to diseased and 04/104 (03.84%) belonged to healthy animals tested. Out of total 90 samples collected from healthy as well as diseased sheep, 13 samples were found culturally positive. From 13 positive samples, 11/43 (25.5%) were diseased and 02/47 (4.25%) were healthy sheep, respectively. Likewise in 118 goats tested, 08/61 (13.11%) resulted positive culturally for pasteurellosis which had clinical infection of respiratory tract and 02/57 (3.51%) healthy goats were also found culturally positive, thusoverall10/118 (08.47 %) goats were found positive for presence of *Pasteurella* sp. (Table 2). The isolates were processed for the confirmation with molecular methods and also typed for expression of some virulence genes.

Phenotypic characterization of *Pasteurella* spp. in sheep and goat

Total 208 samples were collected from diseased as well as healthy sheep and goats. Samples found culturally positive for *Pasteurella* spp. were 23/208 (11.05%), amongst which 16/23 (69.56%) were identified as *P. multocida* and 07/23 (30.43%) were identified as *M. haemolytica*. From the samples of clinically suspected sheep, 11/43 (25.58%) were culturally positive for *Pasteurella* spp. of which 05/11 (45.4%) were *P. multocida* and 06/11 (54.5%) were detected culturally as *M. haemolytica.* In the samples of clinically suspected goats tested for Pasteurellosis, 08/61 (13.11%) were found culturally positive, amongst which 07/08 (87.5%) resulted as *P. multocida* while only 01/08 (14.2) was detected as *M. haemolytica.* From the samples of healthy sheep and goats tested for Pasteurellosis, each 02 isolates from both species were found culturally positive for *P. multocida* (Table 3).

Molecular characterization of *Pasteurella* spp. by PCR

With the advent of biotechnological tools, not only the early and precise identification but also the characterization pertaining to expression of virulence genes in case of bacteria like *Pasteurella* spp. has become possible. In this study, all the total 16 isolates identified as *P. multocida* and 07 isolates of *M. haemolytica* phenotypically were processed by PCR for confirmation. All 16 isolates of *P. multocida* were subjected for KMT gene PCR and also for common capsular antigen specific genes CAP A and CAP D PCR as per standard protocol of Townsend and coworkers (1998 and 2001), respectively. The PCR product of these 16isolates (12 from diseased and 04 from healthy animals) yielded the band of 460 amplicon size of KMT gene upon electrophoresis (Fig. 1) The DNA of these isolates was subjected for expression of capsular antigen by PCR targeting of CAP A and CAP D genes. Out of these isolates of *P. multocida*, CAP A gene generating 1044bp size amplicon size bandswas detected in 13/16 (81.25%) isolates out of which 09/16 (75%) isolates were from diseased and all 04/04 (100%) from healthy sheep and goats ((Fig. 2).

Table.1 PCR Primers for detection of different specific and virulent gene of *P. multocida* and *M. haemolytica*

Gene	Primer and Oligonucleotide sequence	Product length	Reference
KMT	KMT1T7- ATCCGCTATTTACCCAGTGG KMTSP6- GCTGTAAACGAACTCGCCAC	460bp	Townsend *et al.,* (1998)
CAP A	(F) 5'TGCCAAAATCGCAGTCAG 3' (R) 5'TTGCCATCATTGTCAGTG3'	1044bp	Townsend *et al.,* (1998)
CAP D	(F)5'TTACAAAAGAAAGACTAGGAGCCC3' (R)5' CATCTACCCACTCAACCATATCAG3	657bp	Townsend *et al.,* (1998)
PHSSA	(F)TTCACATCTTCATCCTC (R)TTTTCATCCTCTTCGTC	325 bp	Kumar *et.al* (2015)
LKT	(F)5'GCAGGAGGTGATTATTAAAGTGG-3' (R) 5'-CAGCAGTTATTGTCATACCTGAAC-3'	206 bp	Alexander *et al.,* (2008)

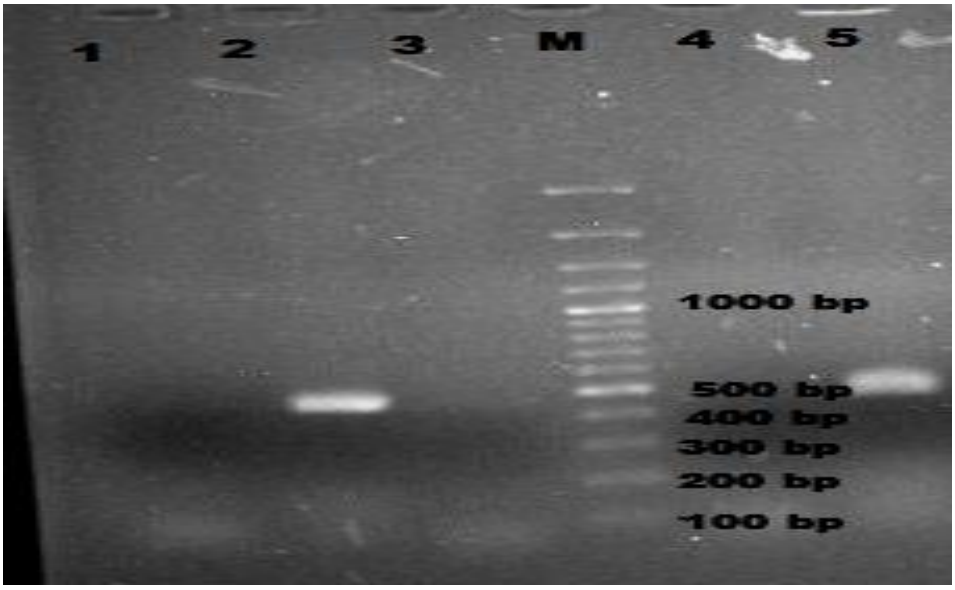
Table.2 Incidence of Pasteurellosis in sheep and goats

Sr no	Animal	Total animal tested	Diseased	Positive		Healthy	Positive		Total	
				No	%		No	%	No	%
1	Sheep	90	43	11	25.58	47	02	04.25	13	14.44
2	Goat	118	61	08	13.11	57	02	03.5	10	08.47
	Total	**208**	**104**	**19**	**18.26**	**104**	**04**	**03.84**	**23**	**11.05**

Table.3 Result of phenotypic characterization of samples from healthy and diseased small ruminants

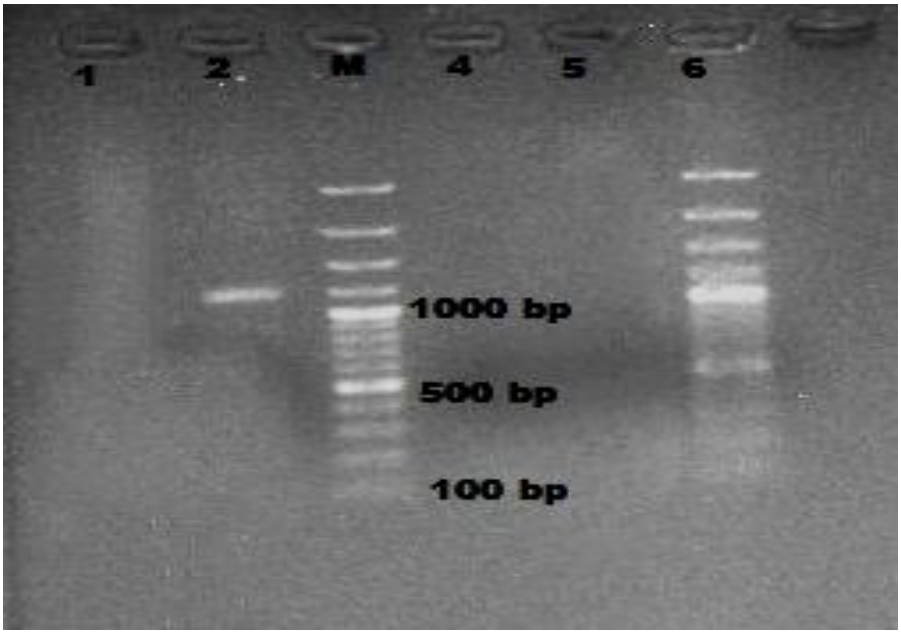
Status of animal	Animal	Total no of samples	Total positive isolates		*P. multocida*		*M. haemolytica*	
			No	%	No	%	No	%
Diseased	Sheep(n=43)	43	11	25.5	05	45.4	06	54.5
	Goat (n=61)	61	08	13.1	07	87.5	01	14.2
Total	**104**	**104**	**19**	**18.2**	**12**	**63.1**	**07**	**36.8**
Healthy	Sheep(47)	47	02	04.25	02	100	00	00
	Goat(57)	57	02	03.5	02	100	00	00
Total	**104**	**104**	**04**	**3.84**	**04**	**100**	**00**	**00**
Grand Total	**208**	**208**	**23**	**11.05**	**16**	**69.56**	**07**	**30.43**

Fig.1 PCR amplified product of 460bp of species specific KMT gene for *P. multocida*



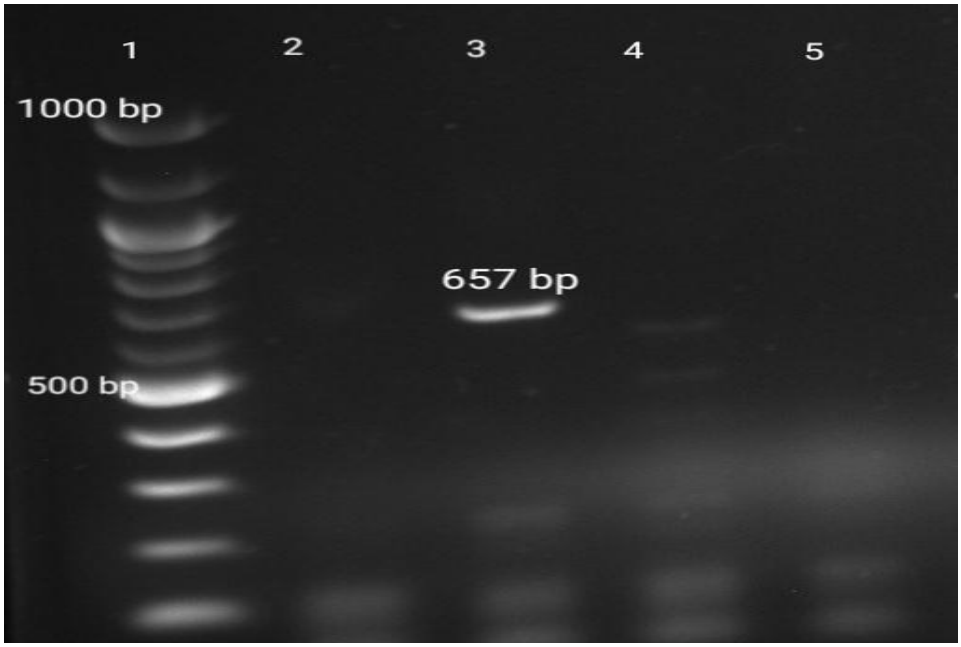
Lane (M) - Marker Ladder of 100 bp size
Lane 2 and 5 - Positive PCR product for KMT gene (460 bp)

Fig.2 PCR amplified product of 1044 bp of capsular antigen CAP A gene for *P. multocida*



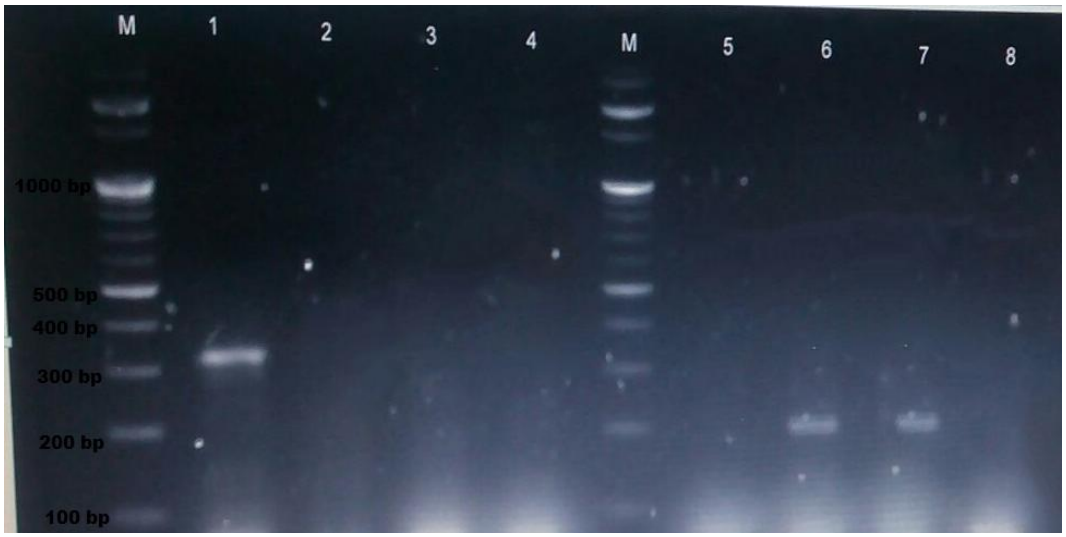
Lane (M) = Marker Ladder of 100 bp size
Lane 2 and 5 = Positive PCR product for CAP A gene (1044 bp)

Fig.3 PCR amplified product of 657 bp of CAP D gene for *P. multocida*



Lane (M) - Marker Ladder of 100 bp size
Lane 3 and 6 - Positive PCR product for CAP D gene (657 bp)

Fig.4 PCR amplified product of 325 bp and 206 bp of PHSSA gene and Lkt gene in *M. haemolytica*



Lane M -Marker Ladder of 100 bp size
Lane 1 - Positive PCR product for PHSSA gene (325 bp)
Lane 6 and 7 -Positive PCR product for Lkt gene (206 bp)

Likewise, CAP D gene generated 657bp amplicon size bands in 08/16 (50.00%) isolates out of which, 07/16 (58.33%) belonged to diseased and only 01/16 (25%) belonged to healthy animals (Fig. 3).

*P. haemolytica*serotype-1 specific antigen (PHSSA) was used to facilitate the rapid detection of virulent *M. haemolytica* and simultaneously its Leukotoxin (Lkt) gene expression was carriedout. Total seven isolates identified as *M. haemolytica* by cultural method were subjected for PHSSA of Kumar and associates (2015) and Lkt gene PCR of Alexander and coworkers (2008), respectively. The desired amplification products of 325 bp and 206 bp were generated upon electrophoresis (Fig. 4).

The results revealed confirmation of 04/07 (57.12%) isolates as *M. haemolytica* with PHSSA gene and 03/07 (42.85%) were positive for Lkt gene, respectively.

The overall incidence of Pasteurellosis was found to be more in sheep than in goats in the present area. The rate of isolation of Pasteurellosis in small ruminants in this study was in agreement with Mohamed and co-workers (2015) but less than that of Tewedros and associates (2015) which may be because of sampling from apparently healthy sheep and goats and also differences in the ecology and managemental practices. *P. multocida* species were isolated and phenotypically identified at higher rate than *M. haemolytica* species from respiratory tract of diseased as well as healthy sheep and goats. *P. multocida* were isolated from diseased as well as apparently healthy sheep and goats indicating they are commensals of respiratory tract while *M. haemolytica* was not isolated in any apparently healthy sheep and goats.

Trend of higher incidence of Pasteurellosis in sheep than goats was reported earlier by Marru and associates (2013) as sheep suffer higher exposure than goats which mostly consume browse (Wilsmore, 2006). *P. multocida* outnumbered *M. haemolytica* in concurrence with results of Karimkhani and associates (2011). All isolates of *P. multocida* were confirmed with PCR that remained in total agreement with cultural method in accordance with Manasa and coworkers (2013) indicating KMT PCR as sensitive and specific alternative for diagnosis and confirmation of it.

However, *M. haemolytica* isolates when processed with and PHSSA and KMT gene PCR almost half of them were not in agreement with phenotypic method. This difference from the outcome of earlier studies of Kumar and associates (2015), might be attributed to the lost polysaccharide capsule by undetected isolates as has been reported by Kirken and Kaya (2003). Capsular antigen PCR of *P. multocida* was suggestive of the fact that CAP A strain was in higher proportion than CAP D strain and in healthy animals CAP A was present in all isolates in agreement with Prabhakar and associates (2012), while CAP D was also found in one isolate. Presence of leukotoxin gene in *M. haemolytica* isolates was observed, hence, confirming the prevalence of its pathogenic strains in small ruminant as reported earlier by Alexander and coworkers (2008), indicating necessity to monitor the emergence of pathogenic *M. haemolytica* strains in future and suggesting more similar studies for the overall assessment for development of area specific effective vaccines.

Acknowledgement

The author is thankful to the Department of Microbiology and Dean, KNP, College of Veterinary Sciences, Shirwal, Dist: Satara, India for providing all the facilities to conduct the present work.

References

Alexander, T. W., Shaun, R. C., Yanke, L. J., Booker, C. W., Morley, P. S., Read, R. R., Gowe, S. P. and McAllister, T. A. (2008) A multiplex polymerase chain reaction assay for the identification of *Mannheimia haemolytica*, *Mannheimia glucosida* and *Mannheimia ruminalis*. Vet Mic130: 165– 175.

Angen, O., Mutters, R., Dominique A., John E. Olsen' and Bisgaard, M.(1999) Taxonomic relationships of the *P. haemolytica* complex as evaluated by DNA-DNA hybridizations and 16s rRNA sequencing with proposal of *Mannheimia haemolytica, Mannheimia granulomatis, Mannheimia glucosida, Mannheimia ruminalis and Mannheimia varigena*. Int. J of Sys Bact (1 999), 49, 67-86.

Babetsa, M., Sandalakis, V., Vougidou, C., Zdragas, A., Sivropoulou, A., Psaroulaki, A. and Ekateriniadou, L. V. (2012). Tetracycline resistance genes in Isolates from Bovine, Ovine, Caprine and Swine Pneumonic Lungs Originated from Different Greek Prefectures. Afr. J. Microbiol. Res.6 (17): 3917-3923.

Deressa, A., Asfaw, Y., Lubke, B., Kyule, M. W., Tefere, G. And Zessin, K. H. (2010) Molecular detection of *Pasteurella multocida* and *Mannheimia haemolytica* in sheep respiratory infections in Ethiopia. Int J Appl Res Vet Med; 8 (2): 101-108

Hawari, A. D., Hassawi, D. S. and Sweiss, M. (2008) Isolation and identification of *Mannheimia haemolytica* and *Pasteurella multocida* in sheep and goats using biochemical tests and Random Amplified Polymorphic DNA (RAPD) analysis.J Bio. Sci. 8(7): 1251-1254

Karimkhani, H., Zahraiesalehi, T., Sadeghizali, M. H., Karimkhani, M. and Lameyi, R. (2011). Isolation of *Pasteurella multocida* from cows and buffaloes in Urmia's Slaughter House. Arc. Razi Institute, 66 (1): 37-41. 2

Kirken, F. and Kaya, O. (2003) Serotyping of *Mannheimia haemolytica* strains Isolated from Pneumonic Lungs of Sheep in the Ayd›n Region of Turkey. Turk J Vet AnimSci 29: 491-494.

Kumar. J., Shivendra Kumar Dixit and Rajiv Kumar. (2015) Rapid detection of *Mannheimia haemolytica* in lung tissues of sheep and from bacterial culture, Vet Wor, Pp. 2231-0916

Manasa, Y. S., Jasni, M. S., Moses, O. O., Maryam Muhammad, E. B and Jsa. Sulaiman. (2013) Isolation and in-vitro antibiotic susceptibility of *Pasteurella multocida* from cattle origin. Int. Res. J. Microbiol, vol. 4: 5.

Mohamed, T. R., Waleed H. Hassan and Wafaa A. Osman. (2015) Isolation, Identification and Antibiogram Studies of *Pasteurella multocida* isolated from Sheep and Goats in Siwa Oasis, Global Veterinaria14 (4): 589-594.

Naveed, M., Javed, M. T., Khan, A. and Krausar, R. (1999) Hematological and bacteriological studies in neonatal lambs with reference to neonatal lamb mortality. Pakistan Vet. J. 19(3): 127-131.

Prabhakar, P., Thangavelu, A., Kirubaharan, J. J. and N. Daniel Joy Chandran (2012) Isolation and characterisation of *P. multocida* isolates from small ruminants and avian origin. Tamilnadu J. Vet & Ani Sciences 8 (3):131-137.

Ricketts, S.W. (1981). Bacteriological examinations of the mare's cervix techniques and interpretation of 454 results. Vet. Rec. 108, 46-51.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning- A laboratory manual. Second Edition. Cold Spring

Harbor Laboratory Press. Pp: 1641-1708.

Tewodros A. Engdaw and Annania T. A. (2015) Pasteurellosis in Small Ruminants: biochemical Isolation, Characterization and Prevalence Determination in Relation to Associated Risk Factors in Fogera Woreda, North-West Ethiopia. Adv in Bio.Res.9 (5): 330-337.

Townsend, K. M., Boyce, J. D., Chung, J. Y., Frost, A. J. and Adler, B. (2001) Genetic organization of *Pasteurella multocida* cap loci and development of a multiplex capsular PCR typing system. J. of Clin.Microbiol39: 924–929.

Townsend, K. M., Frost, A. J., Lee, C. W., Papadimitriou., J. M. and Dawkins, H. J. (1998) Development of PCR Assays for Species- and Type-Specific Identification of *Pasteurella multocida* Isolates. J. Cl. microbiol. 1096–1100

Wilsmore, T. (2006) Diseases of small ruminants in Ethiopia, the veterinary epidemiology and economics research unit school of agriculture's policy and development the university of read, UK. Pp. 67-72.

How to cite this article:

Sneha H. Daphal, Prashant P. Mhase, Mrunalini M. Pawade, Prerana P. Shelke, Jyotica D. Sangle and Rahul P. Kolhe. 2018. Emergence of Virulent *Pasteurella multocida* and *Mannheimia hemolytica* in Sheep and Goats of Western Maharashtra, India. *Int.J.Curr.Microbiol.App.Sci.* 7(09): 1990-1998. doi: https://doi.org/10.20546/ijcmas.2018.709.241