Isolation and Characterization of *Mannheimia varigena* from a Murrah Buffalo

Sarita Yadav[1], Aman Kumar[2], Sushila Maan[2], Ashok Boora[1*], Narender Singh Maan[2], Rajiv Banger[3], K.P. Singh[1] and Inderjeet Singh[1]

[1]*ICAR - Central Institute for Research on Buffaloes (CIRB), Hisar – 125001, India*

[2]*Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar – 125001, India*

[3]*Trainers Training Institute, Hisar– 125001, India*

Abstract: The present study was aimed to isolate and characterize phenotypically as well as genotypically bacterial strain from tracheal froth of a Murrah buffalo on the Government livestock farm (GLF) located at Hisar, Haryana. The isolate showed the cultural, morphological and biochemical properties of *Mannheimia haemolytica* as per Bergey's manual of determinative bacteriology and as confirmed by GENIII microplate (biolog). However, the isolate was further confirmed as *Mannheimia varigena* by 16S rRNA sequencing.

Keywords: *Mannheimia*,16S rRNA sequencing, Murrah buffalo, phenotypic characterization.

INTRODUCTION

Bovine respiratory disease complex (BRDC) is the most economically important multi-factorial disease among ruminants worldwide that is caused by the interaction of host, pathogen and environment [1]. Stress factors such as extreme weather changes, stocking density, transportation and other factors are the precipitating causes to BRDC [2]. The most prevalent infectious agents responsible for BRDC include viral (Bovine herpes virus type-1, Bovine respiratory syncytial virus, Parainfluenza type-3, and Bovine viral diarrheoa virus), bacterial (*Mannheimia haemolytica***,** *Pasteurella multocida***,** *Haemophilus somnus*), and mycoplasmal (*Mycoplasma bovis*) [3-4]. *Mannheimia haemolytica* formerly known as *Pasturella haemolytica* is the primary bacterial agent responsible for BRDC, also referred as bovine pneumonic pasturellosis or shipping fever in which it causes a severe fibrinonecrotic pleuropneumonia [5-7]. The bacterium is normal inhabitant of the upper respiratory tract of healthy ruminants worldwide [8]. However, when animal's immune system becomes compromised following stress and concurrent viral infections, *M. haemolytica* proliferates rapidly and extends downwards into the lungs, where it becomes pathogenic and causes fibrinonecrotic pleuropneumonia [9-10]. This organism is a gram-negative, weakly haemolytic, nonmotile and facultative anaerobic coccobacillus [11]. Historically, *Pasteurella hemolytica* strains were classified biochemically into two biotypes designated A (cattle-associated) and T (sheep-associated) based on their ability to ferment L-arabinose and trehalose respectively [12-13]. Recently, trehalose-negative *P. haemolytica* complex strains (biotype A) were reclassified within a new genus, *Mannheimia*, using studies based on phenotypic data, multilocus enzyme electrophoresis, ribotyping, DNA-DNA hybridization and 16S rRNA sequencing. On the other hand, trehalose-positive strains (biotype T) called as *P. trehalosi* were recently placed into a new genus *Bibersteinia trehalosi* [14]. The genus *Mannheimia* consists of five distinct 16S rRNA clusters (clusters I–V) representing 6 new species of which 5 are named as: *Mannheimia haemolytica* and *M. glucosida* (cluster I), *M. ruminalis* (cluster II), *M. granulomatis* (cluster III), and *M. varigena* (cluster IV) and cluster V comprising unclassified strains [15-19]. While *M. haemolytica, M. granulomatis* and *M. varigena* have been associated with pneumonia, *M. ruminalis* and *M. glucocida* are not of pathogenic significance. Traditional identification of isolates based on phenotypic methods are time consuming and often ambiguous since the genus *Mannheimia* includes very heterogeneous taxa [20-21]. Therefore, genotypic methods involving sequence analysis of the 16S rRNA allow definitive identification of the isolates. Various studies have described the isolation and characterization of *M. haemolytica* from ruminants worldwide including the cattle [22-23], sheep [24] and goat [25] but to date, there has been no reported isolation of this bacterium from buffaloes in India. The present study was aimed to isolate, identify and characterise the bacterium in a sampleof tracheal froth from a dead Murrah buffalo on Government livestock farm at Hisar, Haryana.

*Address correspondence to this author at the ICAR - Central Institute for Research on Buffaloes, Hisar – 125001, India; Tel: +91 1662 281644; Fax: +91 1662 275004; E-mail: ashokcirb@gmail.com

ISSN: 1927-5196 / E-ISSN: 1927-520X/15

MATERIALS AND METHODS

In August and October 2013, twenty-eight elite Murrah buffaloes died suddenly without any premonitory signs of illness over the course of about two weeks at two different occasions on Government livestock farm (GLF) located at Hisar, Haryana.These buffaloes were in their last month of pregnancy and previously vaccinated for FMD and Haemorrhagic septicaemia. During this outbreak, a Murrah buffalo died suddenly in October 2013 and showed respiratory distress including open-mouth breathing and pyrexia (rectal temp 41°C) a day before death. Necropsy revealed fibrinous pneumonia and profuse froth in the trachea indicating towards pasteurellosis. For bacteriological examination, tracheal froth/exudates was taken in sterile container and transported immediately to the laboratory on ice-box. A loopful of tracheal froth was inoculated on blood agar plate (prepared using 5% defibrinated sheep blood) and incubated overnight at 37°C in with the presence of 5% CO_2. The representative colonies were purified through subculturing and subjected to further identification by conventional bacteriological methods involving colony morphology of pure culture on blood agar, gram staining and cell morphology under x100 magnification followed by biochemical tests: catalase (3% hydrogen peroxide), oxidase, indole, urease, citrate, hydrogen sulphide production, nitrate reduction, methyl red and Voges–Proskauer reactions, motility, growth on McConkeyagar, carbohydrate fermentation tests including glucose, mannose, trehalose, mannitol, xylose, dextrin, maltose, lactose, arabinose, melibiose, fructose, sucrose, inositol, arabitol, fucose, ornithine decarboxylase using standard procedures [26]. The isolate was at first identified to species level according to Bergey's manual of determinative bacteriology [27]. To further confirm the identification, standardized biochemical methods using GENIII microplate (biolog) and 16S rRNA sequencing analysis were performed.

Extraction of Genomic DNA (gDNA)

gDNA was extracted from overnight grown broth culture (10 ml) using the Pure link genomic DNA extraction kit (Invitrogen) and according to the protocols in the manufacturer's instructions, with a final elution volume of 50µl. Extracted DNA was stored at -20˚C for further use.

16S rRNA Gene Specific PCR

The 10 µl bacterial gDNA extract and controls (*E. coli*) were amplified with 0.5 µM of each primer (Table **1**), 200 µM of each dNTP (Fermentas), 2X Top Taq buffer master mix (Qiagen). Amplification conditions for both PCRs were as follows: 3 min at 94˚C to denature the DNA, followed by 35 cycles of denaturation at 94˚C for 1 min, primer annealing at 60˚C for 1 min and strand extension at 72˚C for 1 min on a Veriti (ABI) thermal cycler. PCR products were separated on a 1% agarose gel and DNA bands were visualized with ethidium bromide. Amplified PCR products were purified using a gel extraction kit (Qiagen).

Sequencing of PCR Purified Products

Purified PCR products were sequenced in the forward and reverse direction in separate reactions using PCR primers and BigDye terminator v3.1 cycle sequencing kit from Applied Biosystems on an ABI 3130XL genetic analyser.

Sequencing Analysis

The sequence data generated was assembled using SeqMan programme of the Lasergene software ver 5.0. The contig sequence obtained was submitted to Genbank under accession number KM389532 and aligned with the available sequences in the Genbank using MEGA 5.0 programme. The16S rRNA gene sequence of the isolate was compared to already published sequences in the GenBank database using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS AND DISCUSSION

Isolation of Bacterium and its Identification by Conventional Methods:

From tracheal froth, the organism of interest was obtained as a part of mixed flora with *E. coli* and *Klebsiella* probably as post-mortem contaminants. Presumptive colonies were shiny, regular, medium sized with double zone of haemolysis on blood agar (Figure **1**). Gram staining of colonies revealed presence of Gram-negative (G-ve) coccobacilli in the culture (Figure **2**). The colony morphology with double zone of haemolysis and presence of G-vecoccobacilli pointed towards the presence of *Mannheimia haemolytica*or *Mannheimia varigena* in the sample. Biochemical tests were performed on bacterial colony and the results are presented in Tables **1** and **2**. The bacterial colony was found positive in the following tests: β-haemolysis on sheep blood agar, catalase, oxidase, fermentative reaction in Hugh–Leifson medium with glucose, nitrate reduction, acid/acid (A/A)

Table 1: Comparison of Phenotypic Properties of the Isolate Investigated in the Present Study and *Mannheimia haemolytica* as per Bergey's Manual of Determinative Bacteriology [27]

Phenotypic properties	*M. haemolytica*	HSR GLF 132 isolate
catalase	+	+
oxidase	+	+
Nitrate reduction	+	+
ONPG	d	+
Phosphatase	+	+
Gelatinase	-	-
H_2S production	N	-
Ornithine decarboxylase	-	-
Indole production	-	-
Urease	-	-
McConkeyagar growth	+	-
B haemolysis	+	+
Methyl red	-	-
Voges -proskauer	-	-
Lysine decarboxylase	-	-
Acid without gas		
D-Glucose	-	-
L-Arabinose	+	+
Cellobiose		-
Dextrin	+	+
Fructose	+	+
D-Galactose	+	+
Glycerol	d	+
Myo- Inositol	d	+
Lactose	d	+
Maltose	+	+
D-Mannnitol	+	+
D-Mannose	-	-
Melibiose	N	w
Raffinose	d	w
L-Rhammose	d	-
Salicin	N	-
D-Sorbitol	+	w
Trehalose	-	-
D-Xylose	+	+

D: both positive and negative results have been reported for this test.
N: not tested.
W: weak reaction.

on triple sugar iron media (TSI), ONPG (b-galactosidase), phosphatase and production of acid without gas from xylose, dextrin, D-mannitol, D-maltose, D-glucose, D-lactose, arabinose, D-melibiose, D-fructose, and sucrose. Negative results were obtained in the following tests: motility at 37°C,

Figure 1: Isolate grown on blood agar showing colony surrounded with beta hemolytic zone.

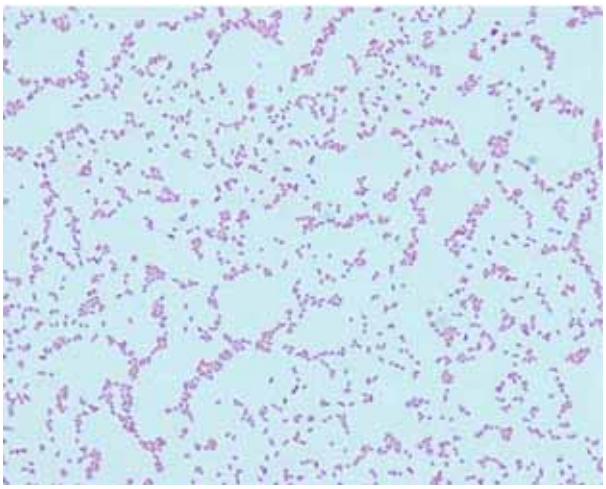

Figure 2: Gram staining of the isolate showing the presence of gram negative coccobacilli.

Simmons citrate utilization, urea hydrolysis, indole production, gelatinase, H2S and gas production on triple sugar iron media (TSI) agar slant, production of acid from D-arabitol, D-fucose, D mannose, trehalose, cellobiose, gentiobiose, methylblue (MB) and Voges Proskauer (VP) reaction, lysine and ornithine decarboxylase, gelatinase reaction.

Testing of sample on McConkey agar yielded a scanty growth of pink colonies. These initial phenotypic characterization based on Bergey's manual of determinative bacteriology revealed that the isolated bacterium belonged to *M. haemolytica.* The standardized biochemical methods using GENIII microplate (biolog) also identified the isolate as *M.*

Table 2: Phenotypic Characterization of the Different Species of *Mannheimia* genus [19]

	M. haemolytica	*M. glucosida*	*M. varigena*	*M. granulomatis*	*M. ruminalis*	**HSR GLF 132 isolate**
Oxidase	+	+	+	+	+	+
Haemolysis	+	+	+	-	-	+
Urease	-	-	-	-	-	-
Ornithine decarboxylase	-	-/+	-/+	-	-	-
Indole	-	-	-/+	-	-	-
D-Xylose	+	+	+	-	-	+
L-Arabinose	**-**	**-/+**	**+**	**-**	**-**	**+**
Mannitol	+	+	+	+	+	+
D-Sorbitol	+	+	-	+	-	+
Maltose	+	+	+	+		+
Trehalose	-	-	-	-	-	-
Dextrin	+	+	+	+	-	+
Raffinose	**+**	**+**	**+**	**-**	**-**	**w**
L-Fucose	+	+	+	+	-	+
L-Rhamnose	-	-	+	-	-	-
Glycerol	**-**	**+**	**+**	**+**	**-**	**+**
Glycosidase*	-	+	-	+	-	-
ONPG	**-/+**	**+**	**-/+**	**-/+**	**+**	**+**

*Glycosidase are cellobiose, gentiobiose and salicin.
Tests in bold indicate similarity to *M varigena.*

Table 3: Primer Sequences Used in PCR

First set of PCR Primer			
Primer	**Sequence (5'-3')**	**Amplicon size**	**Reference**
LPW57	AGTTTGATCCTGGCTCAG	1343	[28]
LPW58	AGGCCCGGGAACGTATTCAC		
Nested set of PCR			
XB1	CAGACTCCTACGGGGAGGCAGCAGT	762	[29]
PSR	ACTTAACCCAACATCTCACGACAC		
PSL	AGGATTAGATACCCTGGTAGTCCA	592	
XB4	GTGTGTACAAGGCCCGGGAAC		

haemolytica. Further, the comparison of biochemical tests of the isolate with published phenotypic reactions could characterise the isolate as *M. varigena* [19] (Table **1**).

PCR Amplification of 16S rRNA

Nested PCR results showed the amplification of right sized bands of 782 and 562 bp using the primer pairs mentioned in Table **3**. The results of amplification are shown in Figure **3**.

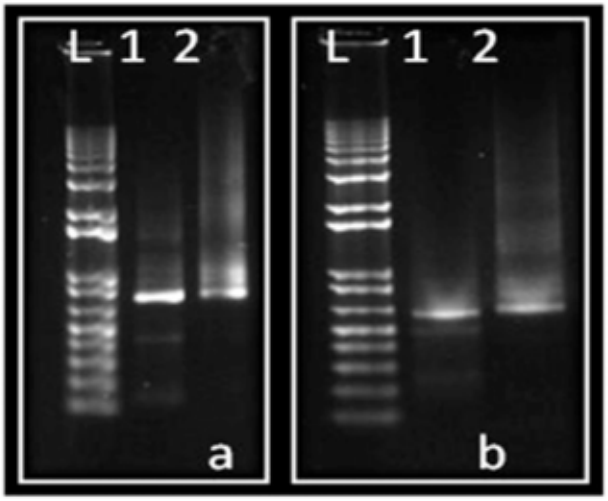

Figure 3: Electropherogram showing amplification of 16S rRNA gene using PSR/XB1 (panel **a**) and PSL/XB4 (panel **b**) primers. Lane L: 100 bp ladder, Lane 1: positive control (*E. coli*) and lane 2: GLF isolate.

Sequence Analysis of 16S rRNA Sequence Data

Sequencing of 16S rRNA amplicons and sequence analysis revealed the presence of *M. varigena* in the sample with 98% nuclueotide identity with the published sequences of *M. varigena*. The results obtained have elucidated the requirement of extensive analysis to precisely identify *Mannheimia* isolates.

CONCLUSION

Defined by typical morphology, growth requirements and metabolic activities together with molecular tools (PCR and 16S rRNA sequencing), the bacterial isolate obtained from tracheal froth of a Murrah buffalo of GLF, Hisar, Haryana has been identified and characterized as *Mannheimia varigena*.

ACKNOWLEDGEMENT

The authors are thankful to the Director, ICAR - Central Institute for Research on Buffaloes, Hisar for all type of facilities to carry out the studies.

REFERENCES

[1] Harhay GP, Koren S, Phillippy AM, McVey DS, Kuszak J, Clawson ML, Harhay DM, Heaton MP, Chitko-McKown CG, Smith TPL. Complete closed genome sequences of Mannheimiahaemolytica serotypes A1 and A6 isolated from cattle. Genome Announcements 2013; 1(3): e00188-13. http://dx.doi.org/10.1128/genomeA.00188-13

[2] Taylor JD, Fulton RW, Lehenbauer TW, Step DL, Confer AW. The epidemiology of bovine respiratory disease: what is the evidence for predisposing factors? Can Vet J 2010; 51: 1095-1102.

[3] Collier JR, Brown WW, Chow TL Microbiologic investigations of natural epizootics of shipping fever of cattle. J Am Veter Med Assoc 1962; 140: 807-10.

[4] Ellis JA The immunology of the bovine respiratory disease complex. Vet Clin Food Anim 2001; 17: 535-549.

[5] Ackermann MR, Brogden KA. Response of the ruminant respiratory tract to Mannheimia (Pasteurella) haemolytica. Microbes Infect 2000; 2(9): 1079-1088. http://dx.doi.org/10.1016/S1286-4579(00)01262-4

[6] Aulik NA, Hellenbrand KM, Kisiela D, Czuprynski CJ. *Mannheimia haemolytica* leukotoxin binds cyclophilin D on bovine neutrophil mitochondria. Microbial Pathogenesis 2011; 50: 168-178. http://dx.doi.org/10.1016/j.micpath.2011.01.001

[7] Gyles CL, Prescott JF, Songer G, Thoen CO. Pathogenesis of Bacterial Infections in Animals 2010; 4th Edition 1-664.

[8] Biberstein EL. The Pasteurelloses. CRC Handbook of Zoonoses 1979; 1(A): 495-514.

[9] Frank GH, Smith PC. Prevalence of Pasteurellahaemolytica in transported calves. Am J Vet Res 1983; 44(6): 981-985.

[10] Omer A, Berhanu A, Chanie M, Fentahun T. Isolation and identification of aerobic bacterial flora in nasopharyngeal passageways of apparently healthy and clinically sick sheep at Gondar University Veterinary Clinic. American-Eurasian Journal of Scientific Research 2012; 7(6): 232-237.

[11] Rice JA, Carrasco-Medina L, Hodgins DC, Shewen PE. *Mannheimia haemolytica* and bovine respiratory disease. Anim Health Res Rev 2007; 8: 117-128. http://dx.doi.org/10.1017/S1466252307001375

[12] Smith GR. Isolation of two types of *Pasteurella haemolytica* from sheep. Nature 1959; 183: 1132-1133. http://dx.doi.org/10.1038/1831132a0

[13] Smith GR. Further observations on the pathogenicity of Pasteurella Haemolytica for young lambs. Journal of Comparative Pathology and Therapeutics 1961; 71: 94-99. http://dx.doi.org/10.1016/S0368-1742(61)80012-X

[14] Blackall PJ, Bojesen AM, Christensen H, Bisgaard M. Reclassification of [Pasteurella] trehalosi as Bibersteiniatrehalosi gen. nov., comb. nov. Int J Syst Evol Microbiol 2007; 57: 666-674. http://dx.doi.org/10.1099/ijs.0.64521-0

[15] Blackall PJ, Angen O, Fegan N, Blackall LL, Mutters R, Bisgaard M. Characterisation of a novel Mannheimiasp from Australian feedlot cattle. Aust Vet J 2001; 79: 634-639. http://dx.doi.org/10.1111/j.1751-0813.2001.tb10787.x

[16] Angen Ø, Aalbaek B, Falsen E, Olsen JE, Bisgaard M. Relationships among strains classified with the ruminant *Pasteurella haemolytica*-complex using quantitative evaluation of phenotypic data. Zentralbl. Bakteriol 1997a; 285: 459-479. http://dx.doi.org/10.1016/S0934-8840(97)80107-7

[17] Angen O, Ahrens P, Bisgaard M. Phenotypicand genotypic characterization of Mannheimia (Pasteurella) haemolytica-like strains isolated from diseased animals in Denmark. Vet Microbiol 2002; 84: 103-114. http://dx.doi.org/10.1016/S0378-1135(01)00439-4

[18] Angen O, Caugant DA, Olsen JE, Bisgaard M. Genotypic relationships among strains classified under the (Pasteurella) haemolytica-complex as indicated by ribotyping and andmultilocus enzyme electrophoresis. Zentralb Bakteriol 1997c; 286: 333-354.

[19] Angen O, Mutters R, Caugant DA, Olsen JE, Bisgaard M. Taxonomic relationships of the [Pasteurella] haemolytica complex as evaluated by DNA-DNA hybridizations and 16S rRNA sequencing with proposal of Mannheimiahaemolytica gen. nov., comb. nov., Mannheimiagranulomatis comb. nov.,Mannheimiaglucosida sp. nov., Mannheimiaruminalis sp. nov.andMannheimiavarigena sp. nov.Int J Sys Bacteriol 1999; 49: 67-86. http://dx.doi.org/10.1099/00207713-49-1-67

[20] Angen O, Olsen JE, Bisgaard M. Further studies of the relationships among strains classified as taxon 15, taxon 18, taxon 20, (Pasteurella) granulomatis or the (Pasteurella) haemolytica-complex in ruminants using quantitative evaluation of phenotypic data. Zentralbl Bakteriol 1997b; 286: 317-332. http://dx.doi.org/10.1016/S0934-8840(97)80090-4

[21] Angen O, Quirie M, Donachie W, Bisgaard M. Investigations on the species specificity of Mannheimia (Pasteurella) haemolytica serotyping. Vet Microbiol 1999b; 65: 283-290. http://dx.doi.org/10.1016/S0378-1135(98)00304-6

[22] Haji Hajikolaei MR, Ghorbanpour M, SeyfiabadShapouri MR, Rasooli A, Ebrahimkhani D, Jabbari AR. Bacteriological and serological studies on Mannheimiahaemolytica infection in cattle slaughtered at Ahvaz (southwestern Iran) abattoir. Iranian Journal of Veterinary Research, Shiraz University 2010; 11(1): 84-87.

[23] Kaliek AE, Mokhtar AAI, Selim A, Rizk MK. Bacterial isolates from calves slaughtered at abattoir suffering from respiratory problems in Sharkia Governorate. World Rural Observations 2013; 5(1): 47-51.

[24] Kirkan S, Kaya O. Serotyping of Mannheimiahaemolytica strains isolated from pneumonic lungs of sheep in the Aydin region of Turkey. Turk J Vet AnimSci 2005; 29: 491-494 .

[25] Horadagoda NU, De Alwis MCL, Wettimuny SG DES, Vipulasiri A. Biochemical and cultural characterization of Pasteurellahaemolytica isolated from pneumonic lungs of goats. J Natu Sci Coun Sri Lanka 1994; 22(4): 325-333.

[26] Quinn PJ. Pasteurella species. In: Quinn PJ. Carter ME, Markey BK, Carter GR, Eds. Clinical Veterinary Microbiology. Mosby, Edimburgh 1994; 254-259.

[27] Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Bergey's Manual of Determinative Bacteriology 1994; Ninth edition.

[28] Woo PCY, Cheung EY, Leung K, Yuen K. Identification by 16S ribosomal RNA gene sequencing of an Enterobacteriaceae species with ambiguous biochemical profile from a renal transplant recipient. Diagn Microbiol Infect Dis 2001; 39: 85-93. http://dx.doi.org/10.1016/S0732-8893(01)00206-1

[29] Xu J, Smyth CL, Buchanan JA, Dolan A, Rooney PJ, Millar BC, Goldsmith CE, Elborn JS, Moore JE. Employment of 16 S rDNA gene sequencing techniques to identify culturable environmental eubacteria in a tertiary referral hospital. J Hosp Infect 2004; 57: 52-58. http://dx.doi.org/10.1016/j.jhin.2004.01.011

Received on 20-03-2015 Accepted on 24-03-2015 Published on 03-04-2015

DOI: http://dx.doi.org/10.6000/1927-520X.2015.04.01.3