

Molecular Detection of *Pasteurella multocida* and *Mannheimia haemolytica* in Sheep Respiratory Infections in Ethiopia

A. Deressa¹

Y. Asfaw^{2*}

B. Lubke³

M. W. Kyule⁴

G. Tefera⁵

K.-H. Zessin⁶

¹Ethiopian Health and Nutrition Research Institute, Addis Ababa, Ethiopia,

²Addis Ababa University, Faculty of Veterinary Medicine, P. O. Box 34, Debre Zeit, Ethiopia,

³Institut für Mikrobiologie und Tierseuchen, Philippstr.13, D-10115 Berlin, Germany

⁴Freie Universität-Berlin, Postgraduate Studies in Tropical Veterinary Epidemiology, Luisenstrasse 56, 10117 Berlin, Germany

⁵Institute of Biodiversity Conservation, Addis Ababa, Ethiopia.

* corresponding author: Email:yilkalafaw2005@yahoo.com

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ABSTRACT

The polymerase chain reaction (PCR) was used to detect *Pasteurella multocida* and *Mannheimia haemolytica* directly from clinical and section tissues of acute respiratory diseases of sheep. Laboratory specimen consisting of nasopharyngeal swab, tonsil and lung was collected from two types of sheep: Menz local and Black Head Somali sheep in selected areas of Ethiopia. A total of seventy-four specimens were used for the detection of the species directly. The strains of *P. multocida* and *M. haemolytica* were detected from 20 specimens. Both *P. multocida* and *M. haemolytica* were detected by PCR assay in this study. *P. multocida* was

detected from nasopharyngeal swabs (n=3) and lung (n=3); this species, however, was not detected from the tonsil. *M. haemolytica* was detected from all the three specimens (nasopharyngeal swabs 2, tonsils 2, and lungs 10). Besides, *P. multocida* capsular type A tox⁺ and *P. multocida* capsular type D tox⁺ were observed from lung and nasopharyngeal swabs. As it was observed in this study, PCR assay when performed on specimens taken directly from clinical and section tissues represents species specific method for detection of *P. multocida* and *M. haemolytica* in sheep.

INTRODUCTION

P. multocida and *M. haemolytica* are the known bacterial pathogens, which cause severe respiratory diseases of sheep and cattle^{3,5}. In recent years, genotypic methods,

Table 1. -The primer pairs used in PCR for *P. multocida* and *M. haemolytica*

Bacterial species	Name of Primer	Number of Primer	Sequence	%GC	Source of Primer pair	Length of the amplification product (bp)
<i>P. multocida</i>	KMT-1SP6	T2248A05	GCTGTAACGAACTCGCCC	55	[15]	460
	KM-TIT1	T2248A06	ATCCGCTATTTACCCAGTGG	50		
<i>M. haemolytica</i>	Rpt2	T2967B03	GTTTGTAAGATAATCCCAITTT	30	[10]	1022
	Rpt2 rev	T2967B04	CGTTTTCCACTTGGCGTGA	50		

Table 2 - Oligonucleotides used for the amplification of species-specific and virulence associated genes of *Pasteurella* and *Mannheimia* species

Oligonucleotides	Size of product (bp)	Gene	Reference	Sequence reference	Species
KMT1/SP6T7	460	KMT1-sequence	[15]	[15]	<i>P. multocida</i>
PomA fwd/rv	921	pomA	IMT	[16]	<i>M. haemolytica</i>
ToxA fwd/rv	866	toxA	IMT	[9]	<i>P. multocida</i>
Synth ewd/rv	1264	pmHAS	IMT	[4]	<i>P. multocida</i>

especially nucleic-acid based assays, allow the detection of microorganisms by dramatically improving the sensitivity and decreasing the time required for bacterial identification⁸. (*P.*) *haemolytica* *trehalose-negative* is reclassified as *Mannheimia haemolytica* comb. Nov., (*P.*) *granulomatis* Bisgaard taxon 20 is reclassified as *Mannheimia granulomatis* comb. Nov. and (*P.*) *haemolytica* biovar 3 is reclassified as *Mannheimia glucosida* sp. Nov.¹. In sheep, respiratory infections and pneumonia caused by *Pasteurella* pathogens is prevalent in Ethiopia¹¹. All prevalence studies were carried using standard bacteriological and serological techniques; to date no PCR assay has been used for detection of *Pasteurella* pathogens in Ethiopia. The present study was carried out to detect *Pasteurella* pathogens involved in sheep respiratory infections using PCR assay.

MATERIALS AND METHODS

Sampling and Transporting

Specimens were collected from seventy-four sheep with acute respiratory infection and acute bronchopneumonia or pneumonia from October 2003 to March 2004 from Debre Birhan, Harshin, and Jijiga areas in Ethiopia (Map I). The specimens were collected from two types of sheep: Menz local and Black Head Somali sheep. From this total number, thirty-seven were taken from the lung, ten from tonsil and twenty-seven were nasopharyngeal swabs. The specimens were transported to the National Veterinary Institute (NVI) Laboratory using the modified Cary-Blair Medium, which is special for *P. multocida* and *M. haemolytica*¹².

Reference Strains

Ten lyophilized reference strains of *P. haemolytica* including A1 (P588), A2 (P499), A5 (P509), A7 (P503), A8 (P504), A9 (P505), A11 (P507), A12 (P501), A13 (P509) and T15 (P511) kindly provided by BgVV (Germany) and *P. multocida* strain 14381 provided by NVI (Ethiopia) were used.

Culturing

All field and reference strains were grown on sheep blood agar plates at 37°C for 24h. Isolation of *P. multocida* and *M. haemolytica* was done using Tryptose soy broth and blood agar as primary isolation media following standard procedures. Camp test and ENTERO Rapid 24 hour biochemical kit were used for identification¹³. Colonies giving gram-negative coccobacilli or short rods with or without bipolar staining on smears were subcultured for identification. A 24-hour pure *Pasteurella* suspected culture (isolate) was also subjected to biochemical tests using standard procedures. Mixtures of pure culture colonies (i.e. 4: 4 different colonies) were used for preliminary observation by PCR assay. Reference strain of *Pseudomonas aeruginosa* was used for negative control.

Standard Solutions and Reagents for PCR

10x PCR Buffer, deoxynucleotides (dNTPs), Taq polymerase (BIOTOOLS, Spain) and primers (Table I) (Invitrogen Ltd, Paisley, Scotland) were used for multiplex PCR assay. Genomic DNA template was prepared using Invisorb™ Genomic DNA kit III (Invitex, Germany). This kit was used according to the manufacturer's protocol to prepare genomic DNA from clinical and section materials.

Master Mix of PCR

The mix for PCR was prepared using 1x PCR buffer (50mM KCl, 10mM Tris-HCl [pH8.3], 1.5mM MgCl₂), 0.2mM dNTP, 0.5μM each primer of *P. multocida* and 2μM each primer of *M. haemolytica*, 2 units of Taq polymerase and 1μg of DNA template to have a final volume of 25μl.

PCR Assay

Polymerase chain reaction was performed on a PTC-200 Pettier Thermal Cycler (MJ Research), with an initial denaturation temperature of 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 30sec.

Table 3 - Results of PCR analysis for *P. multocida* and *M. haemolytica* in comparison with standard diagnostic methods

Characteristics		Analyzed specimens								
		Swabs (n=27)			Tonsil (n=10)			Lung (n=37)		
		Pm	Mh	N	Pm	Mh	N	Pm	Mh	N
PCR ¹		3	2	22	0	2	8	3	10	24
Growth on sheep blood agar, 24 h	round and grayish with a musty or mushroom sort of smelly colonies (some variants were more opaque), non-hemolytic	+	-	-	+	-	-	-	-	-
	circular, glistening colonies, narrow double-zone hemolysis	-	+	-	-	+	-	-	+	-
Growth on MacConkey agar, 24 h	none	+	-	-	+	-	-	-	-	-
	pink to red colonies	-	+	-	-	+	-	-	+	-
Growth on selective medium for Pasteurella and Mannheimia, 24 h		+	+	-	+	+	-	-	+	-
Gram- negative	coccobacilli numerous, bipolar stain marked	+	-	-	+	-	-	-	-	-
	evenly stained rods numerous	-	+	-	-	+	-	-	+	-
CAMP test		-	+	-	-	+	-	-	+	-
Indole+ trehalose-, 8 h (rapid)		+	-	-	+	-	-	-	-	-
Indole -, trehalose+, 8 h (rapid)		-	+	-	-	+	-	-	+	-

¹ = the PCR result indicates the number of specimens

Pm = number of specimens from which only *P. multocida* were detected,

Mh= number of specimens from which only *M. haemolytica* were detected,

N= number of specimens from which *P. multocida* and *M. haemolytica* were not detected

Gel Analysis of the PCR Products

The products of PCR were separated by electrophoresis on 2% agarose gel (SIGMA-ALDRICH CHEMIE GmbH, Germany) in 1x TAE (0.04 M Tris-Acetate; 0.001 M EDTA [pH 8.0]) buffer at room temperature using gradients of 10V/cm. For gel analysis, 10µl of the products was loaded in each gel slot. A 100bp DNA Ladder (Promega) was used to determine the fragment sizes.

Finally, results were visualized after staining the gel in 0.5µg/ml ethidium bromide.

RESULTS

The specificity of the primer pairs, which were used for PCR, were verified separately by PCR assay. Pure culture colonies of 10 reference and 20 field strains of *P. multocida* and *M. haemolytica* isolated from sheep in Ethiopia were used for analysis. All the strains of both bacterial species gave posi-

Figure 1. - Molecular identification of *P. Multocida* subspecies and biovar 2 of *P. avium* and *P. canis*

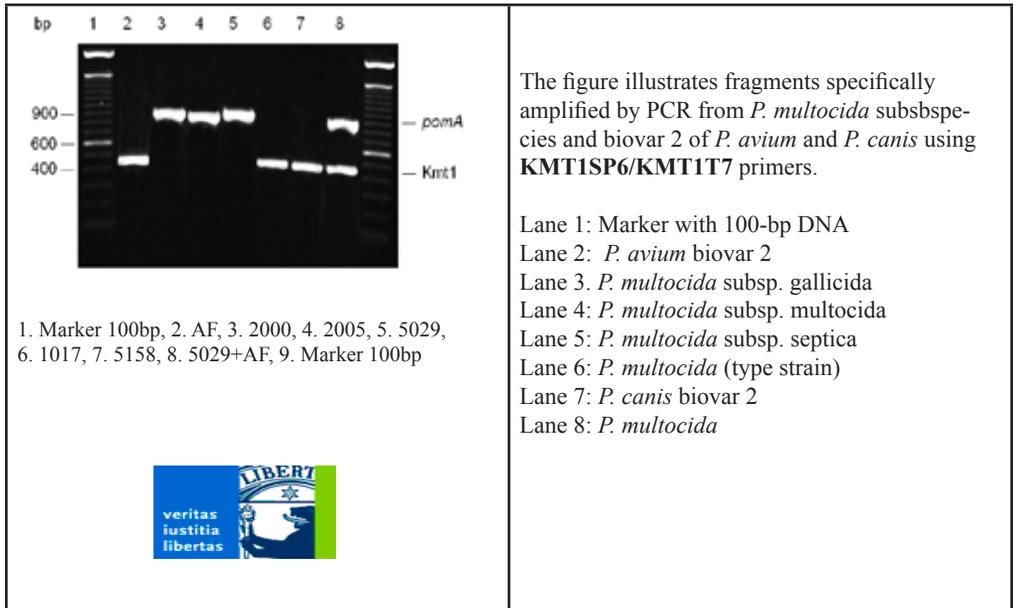
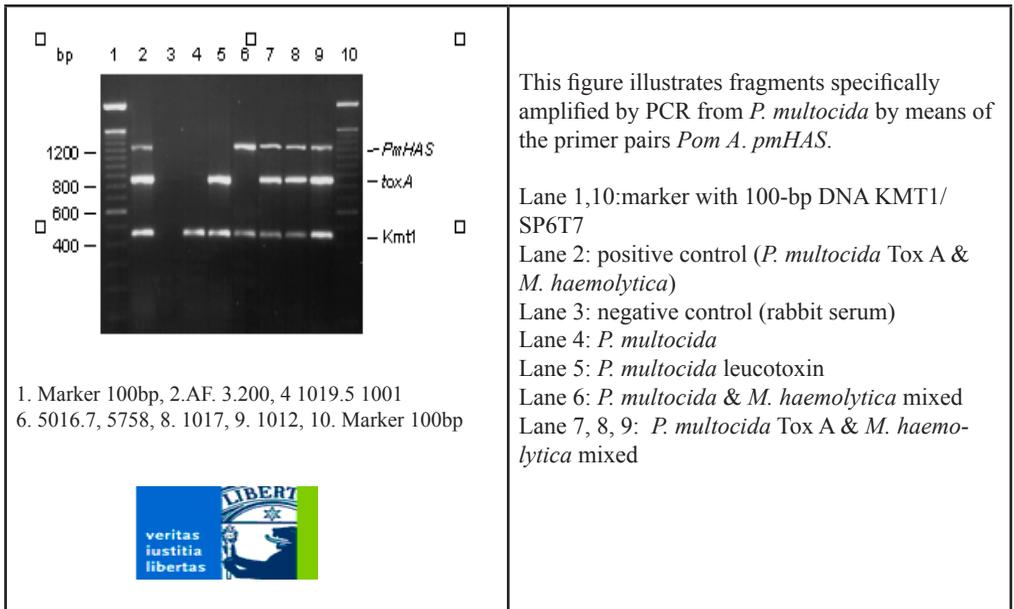


Figure 2. - Molecular characterization *P. Multocida*



tive reaction with specific amplifications. The amplification results of this preliminary observation are shown (Figure 1).

The mixtures of pure culture colonies of the strains of *P. multocida* and *M. haemolytica* were used for optimization of the PCR assay. The standard PCR results were fully correlated in both bacterial species.

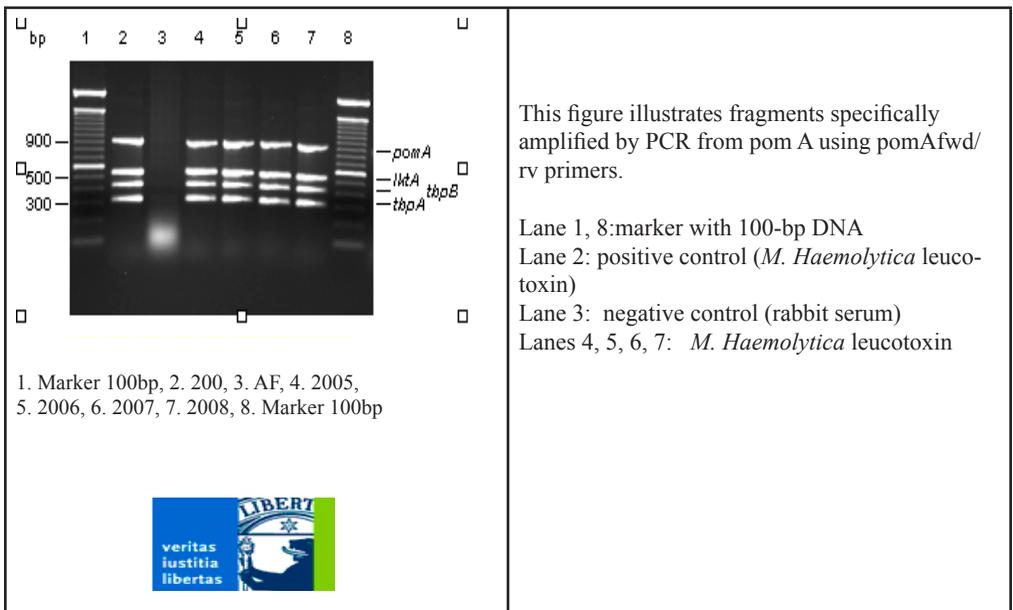
A total of 74 field specimens (27 nasopharyngeal swabs, 37 from lung, and 10 from tonsil) were used for detection of *P. multocida* and *M. haemolytica* strains by PCR assay. From these specimens PCR assay showed 20 positive results. Both *P. multocida* and *M. haemolytica* were detected in this study. *P. multocida* was detected from nasopharyngeal swabs (n=3) and lung (n=3); this species, however, was not detected from the tonsil. *M. haemolytica* was detected from all the three specimens (nasopharyngeal swabs 2, tonsils 2, and lungs 10). The PCR results observed using pure culture colonies, and direct clinical specimens and section materials were correlated with no difference (Table III). The oligonucleotides used for the amplification of species-specific and virulence associated genes of Mannheimia and Pasteurella species were shown (Table

II). The amplification of *P. multocida* and *M. haemolytica* strains taken from clinical specimens and section materials is shown in figures 2 and 3.

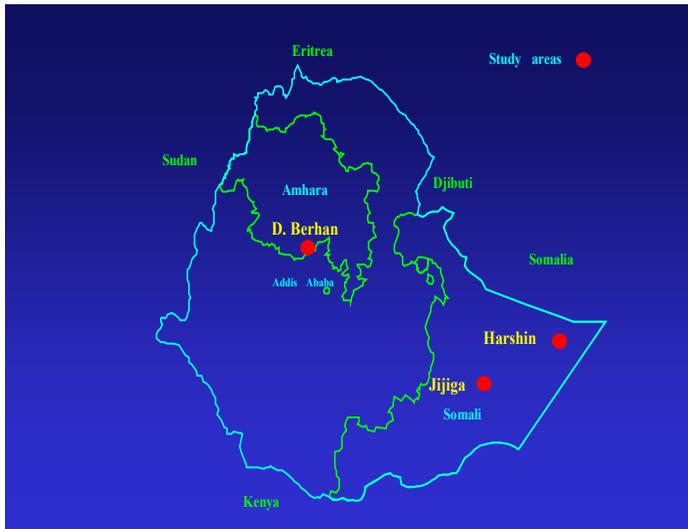
DISCUSSION

The amplification of the reference and field strains, during the preliminary study, was very specific to their species. This confirmed the specificity of PCR with accurate results. Furthermore, it was observed that the primer pair KMT1SP6/KMT1T7¹⁵ amplified a product of 460 bp from all strains of *P. multocida* with all its subspecies (*P. multocida* subsp. gallicida, *P. multocida* subsp. multocida, *P. multocida* subsp. septica) and from biovar 2 of *P. avium* and *P. canis*. Both strains were reported from pneumonic calves in several countries². The 16S rRNA sequence studies of members of the family Pasteurellaceae indicated that taxon 13 of Bisgaard, under which both biovars were originally classified as *P. multocida*-like strains, should be reinvestigated. Recently, it was found that the 16S rRNA similarities between *P. multocida* and biovar 2 of *P. avium* and *P. canis* are greater than 98.6%⁷. This is also observed by comparative analysis of 220 bp fragment of DNA of *P.*

Figure 3. - Molecular characterization *M. Haemolytica*



Map 1. - Selected study areas in Ethiopia



multocida, biovar 2 of *P. avium* and *P. canis*. On this background it appears that the DNA amplification of both strains is not a false-positive, rather it indicates a higher degree of genomic relatedness of both biovars to *P. multocida* than was previously observed by DNA-DNA hybridization¹⁴. On the other hand, the primer pair Rpt2/Rpt2 rev¹⁰ amplified a product of 1022 bp from all strains of *M. haemolytica*.

The main emphasis in the present study was, however, the development of a rapid and multiple detection method for *P. multocida* and *M. haemolytica* in sheep, especially during acute cases of respiratory diseases. However, to our knowledge no PCR assay has been used for detection of both species directly from clinical specimens in sheep. LICHTENSTEIGER et al.⁶ used PCR for detection of toxigenic *P. multocida* directly from swab specimens in pigs showing that direct specimen analysis using PCR is more rapid and specific than standard methods of identification, hence, facilitating clinical diagnosis. The strains of *P. multocida* and *M. haemolytica* were successfully detected directly from clinical and section materials. All the specimens which were used for PCR were examined based on their colonial and cellular morphology and biochemical reactions. In this study, the results of the

PCR assay and the standard method showed complete agreement.

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