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Research Article

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Molecular Detection and Genotyping of *P. Multocida*, *M. Hemolytica*, and *B. Trehalosi* Isolates Targeting the Virulence Associated Genes from Ethiopian Cattle and Sheep

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Abstract

Pasteurellaceae families are usually considered opportunistic pathogens which inhabit normal flora on the mucosal membranes of the upper respiratory and the lower genital tracts of mammals and birds. The majority of P. multocida, M. hemolytica, and B. trehalosi isolates are opportunistic animal pathogens and cause disease only under certain conditions. Some of the target genes are shared by isolates found in the same or different genera.. The alpha / beta hydrolase kmt1 genes part of OMP and SLP are shared by all P.multocida species and FbpA is common gene for both P.multocida and M. hemolytica isolates. Other virulence related genes are used for genotyping of P.multocida, M.hemolytica and B.trehalosi isolates. The objective of the current research was focused on the detection and genotyping of different target genes that can be found in the isolates of P. multocida, M. hemolytica, and B. trehalosi using various PCR primers. The distributions of virulence-associated genes were assessed in the isolates of the three genera.

Materials and methods

A total of eight isolates from P. multocida, M. hemolytica, and B. trehalosi isolated from clinical cases of cattle and sheep and stored at the Bacteriology laboratory of NVI were used as source of samples for the present study. DNeasy® Blood & Tissue Kit (Qiagen, Germany) was used for the extraction of DNA from bacterial isolates. Different primers were used for genotyping of P. multocida, P.hemolytica and B.trehalosi isolates.

Results

P.multocida was detected using species-specific kmt1 primer with an amplicon product of 460bp. Four serogroups of P.multocida (A, B, D, &E) were detected using serotype-specific primers with amplification products of 1044bp, 760bp, 657bp, and 511bp respectively. PCR was conducted to HS causing P.multocida type B and detected 620 bp amplicons. SLP genes were detected in all P.multocida serotypes with an amplification product size 1400-1553bp. In addition to that, the four P. multocida serotypes were found to be positive for BRD-PmSLP with an amplification product of 460bp but using recombinant HS-SLP primers resulted in the double band at 460 bp and 541bp for serotypes A and D. However, HS causing type B& E serotypes were detected with an amplified product of 541bp. TbpA2 has been detected with an amplicon size of approximately 750 bp for types (A and D) and 1300bp for type B using TbpA-F2/Rev primers. All of the P.multocida serotypes (A, B, D&E) and M. hemolytica (A: 1) were strongly positive for the iron acquisition FbpA gene with amplification bands at 500bp and 1000bp, respectively. A multiplex PCR as-

say was carried out for detection of M. haemolytica A: 1 using PHSSA and Rpt2 primers with amplification products of 327pb and 1022 bp respectively. The three isolates of B. trehalosi were identified using BtsodA primers with the result of 144bp amplicon size.

Conclusion

From this work, we understood that P.multocida and M. haemolytica shared genetic materials like iron binding proteins (FbpA) and some of the target genes (kmt1 and SLP) were commonly found in all isolates of P. multocida. However, TbpA2 was found to be in most but not in all Pasteurella isolates. We also observed that PHSSA and Rpt2 genes were exclusively found in M. hemolytica isolates whereas Bt-sodA was prominent in all B. trehalosi isolates (T3, T4&T15).

Keywords: Biberstenia Trehalosi, Mannhemia Hemolytica, Pasteurella Multocida, PCR, Serotype, Virulence gene, Genotype

Introduction

Members of the *Pasteurellaceae* family are usually considered opportunistic pathogens which inhabit normal flora on the mucosal membranes of the upper respiratory and the lower genital tracts of mammals and birds [1]. The majority of *P.multocida, M.hemolytica,* and *B,trehalosi* isolates are opportunistic animal pathogens and cause disease only under certain conditions [2, 3].

P. multocida is a multi-host species and an important animal health pathogen that causes several diseases like hemorrhagic septicemia in cattle, enzootic bronchopneumonia in cattle, sheep, and goats, and atrophic rhinitis in swine, fowl cholera in poultry, and snuffles in rabbits [4, 5]. Members of the genus *Mannheimia* produce several biological substances that are associated with pathogen virulence. They required a higher concentration of iron for the production of cytotoxin than needed for growth. *M. hemolytica* is the most virulent, prominent, and highly pathogenic microorganism that causes very severe respiratory diseases in ruminants [6]. It is one of the primary bacterial agents responsible for bovine pneumonic pasteurellosis (shipping fever) because of its frequent occurrence in transported animals [7]. *Bibersteinia trehalosi* is an important pathogen that causes septicemia and respiratory disease in calves and lambs [8].

P.multocida subdivided into *P. multocida subsp. multocia, P. multocia* subsp. *gallicida and P. multocida* subsp. *septica* based on their acid production differences from dulcitol (–)-D-sorbitol [9]. *P. multocida* subsp. *multocida* isolates can ferment sorbitol but not dulcitol; *P. multocida* subsp. *septica* isolates can't ferment both sorbitol and dulcitol, whereas the *P. multocida* subsp. *gallicida* isolates can ferment these two chemicals [10]. P.multocida has been classified into five serogroups designated as A, B, D, E, and F depending on their capsular antigens [11, 12]. An indirect *haemagglutination* (IHA) and gel diffusion precipitation (GDP) tests have been used for capsular serotyping of *P. mutpocida* [11, 13 and 14].

Virulent isolates of *P. multocid*a can produce capsular antigens which are closely associated with lipopolysaccharides [8]. The antigenic properties of these capsular polysaccharides are used for typing *P. multocida* isolates [15]. The major polysaccharide component of the capsule in serogroup A is hyaluronic acid which is sensitive to hyaluronidase [6, 11]. The capsular components of serogroup B isolates are arabinose, mannose, monosaccharide, and galactose [8]. The capsular material of serogroup D isolates shows similarities with hyaluronic acid. However, it is not susceptible to hyaluronidase. The capsular group D also contains heparin which is susceptible to heparinase III and chondroitinase AC. Type D isolates can be identified using an acriflavine test assay.

The capsular substance of serogroup F isolates contains chondroitin-sulfates that are susceptible to chondroitinase AC, but not to hyaluronidase/heparinase III [15]. Serotypes B and E are the only *P. multocida* serotypes with hyaluronidase activity but the chemical composition of the serogroup E capsule remains unknown [17].

P. multocida serotypes are separated into 16 somatic serovars based on lipopolysaccharides. Somatic serotypes were recognized based on the gel-diffusion precipitin and tube agglutination testing methods [12,13]. In the designation of isolates, the letter represents the capsular antigens and the number stands for the somatic antigens. For example, the newer designations of serogroup B: 6 and E: 6 are based on the Namioka-Carter classification system. These two serotypes were formerly designated as B: 2 and E: 2, respectively, according to the Carter Heddleston system of classification [18]. The former *Pasteurella hemolytica* biotypes A (L-arabinose fermenter) and T (trehalose fermenter) were reclassified as new genera Mannheimia and Bibersteinia, respectively [19]. Based on capsular polysaccharide typing by the IHA test, thirteen biotypes A isolates (A:1, A:2, A:5-A:9, A:11, A:12-A:14, A:16, and A:17) and four biotype T isolates (T:3, T:4, T:10 & T:15) have been identified [16, 20 and 22]. Out of 13 *P. hemolytica* biotype A isolates, 12 serotypes were renamed as *M. hemolytica,* and A: 11 was classified into a new taxon *M. glucosida* due to differences in biochemical profile. Biotype T isolates were named *Bibersteinia trehalosi* [23].

Pasteurella and *Mannheimia* species can be distinguished by colonial and growth characteristics, and by biochemical reactions. Isolates of *P. multocida* can be differentiated by serotyping and

biotyping, whereas *M. hemolytica* and *B. trehalosi* isolates are usually differentiated by serotyping [24]. *P. multocida* strain serotyping was conducted using capsular type antisera by rapid slide agglutination test Meanwhile, *M. hemolytica* isolates were serotyped using a rapid plate agglutination procedure as described by Frank and Wessman [24, 25 and 27]. However, there were difficulties in the use of a more laborious indirect hemagglutination test, limitations of specificity, and traditional phenotypic procedures.

Many rapid molecular detections and identification methods have been developed for differentiating *Pasteurella, Mannhemia,* and *Biberstenia* isolates to overcome problems related to IHA tests and non-serologic tests [28, 29 and 31]. PCR assays allow the detection of organisms directly from clinical samples or cultured bacterial cells, thus dramatically improving the sensitivity and reducing the time required for bacterial identification. PCR capsular typing has been developed for *P. multocida* isolates and it is possible to detect virulence-associated genes in all *Pasteurella, Mannhemia,* and *Bibersteinia* isolates using this method [3].

The current study was focused on molecular genotyping of *Pasteurella, Mannhemia,* and *Bibersteinia* species using the virulence associated gene primers at the National Veterinary Institute (NVI).

Materials and Methods

Bacterial Isolates

A total of eight bacterial isolates obtained from the Bacteriology laboratory of the National Veterinary Institute (NVI) were used as a source of samples for this study.

Table 1: Bacterial isolates included in the present study

Sample type	Host of origin	Selected isolates	Reference
Fresh culture	Bovine	*P. multocida type A:1*	[32]
Blood &Tissue suspension	Bovine	*P. multocida type B:6*	
Fresh culture	Bovine	*P. multocida type E*	
Fresh culture	Bovine	*P. multocida type D*	
Fresh culture	Bovine	*M. hemolytica A:1*	
Fresh culture	Ovine	*B. trehalosi* T_3, T_4 & T_{15}	

Extraction of DNA from Bacterial Isolates

Bacterial genomic DNA from all the isolates was extracted using DNeasy® Blood & Tissue Kit according to the manufacturer's instructions (QIAGEN, Germany).

Primers and PCR conditions

Various set of primers for *P. multocida* (kmt1, capA, capB, clone 6b, capD, capE, & *Pm*FbpA), *M. hemolytica* (MhFbpA) and *B. trehalosi* (BtsodA) provided by the University of Calgary (Canada). *P. multocida* virulent associated gene primers (TbpA2 and PmSLP) supplied by the University of Toronto (Canada), and *M. hemolytica* specific primers (Rpt2 and PHSSA) provided by VBC Biotech (Austria) were used in the present study.

PCR reaction mixtures are optimized based on the instruction of the detection kit manufacturer (Lot: EP0703, Thermo Scientific, Thermo Fisher Scientific Inc.).

For most PCR reactions, 5µl template DNA was added to the total 20 µl reaction mixture containing 4.5µl of nuclease-free water, 2µl of each forward and reverse primer pair (5pmol/µl), 5 µl of 10x DreamTaq™ Buffer that contains 20mM MgCl2, 5µl of dNTP Mix (2mM each) and 1.5µl of Dream Taq™ polymerase

Amplification protocols for the capsular biosynthesis gene of *P. multocida,* PHSSA-1, and methylene transferase encoding gene(Rpt2) of *M. hemolytica* and manganese-dependent superoxide dismutase encoding gene of *B. trehalosi* (BtsodA) were used as previously described by respectively [29, 33 and 34]. PCR thermal cycling conditions for the iron acquisition TbpA2 and FbpA genes were also prepared as previously stated by respectively [35, 36]. PCR instructions provided by the University of Toronto were used as a PCR protocol for surface lipoproteins (PmSLP) locus [37]. The primer sequences and amplicon size are clarified in Table 2. The PCR thermal cycling conditions of the primers are Shown in Table 3.

PCR amplification was performed using the thermal cycler 2720 (Applied Biosystems, USA) for each PCR work. PCR products were analyzed by gel electrophoresis on a 1.5% agarose gel stained with PRONASAFE nucleic acid staining solution produced by Condalab (Spain) and analyzed using a 100bp DNA ladder M (Hi-Media, India), and photographed using UV transilluminator (UV Tech, France).

Table 2: Primers used for detection of different target genes of *P. multocida, M. haemolytica, and B. trehalosi* isolates from NVI

Target gene	Primer name	Primer sequence 5'-3'	Amplicons (bp) size	Reference
kmt1	KMT1T7	ATCCGCTATTTACCCAGTGG	460	[33]
	KMT1SP6	GCTGTAAACGAACTCGCCAC		
hyaD-hyaC	capA-F	GATGCCAAAATCGCAGTCAG	1044	[28]
	capA-R	TGTTGCCATCATTGTCAGTG		
bcbD	capB-F	CATTTATCCAAGCTCCACC	760	[28]
	capB-R	GCCCGAGAGTTTCAATCC		
dcbF	capD-F	TTACAAAAGAAAGACTAGGAGCCC	657	[28]
	capD-R	CATCTACCCACTCAACCATATCAG		
ecbJ	capE-F	TCCGCAGAAAATTATTGACTC	511	[28]
	capE-R	GCTTGCTGCTTGATTTTGTC		
B:6	KTT72-F	AGG CTCGTTTGGATTATGAAG	620	[33]
	KT SP61-R	ATC CGC TAA CAC ACTCTC		
FbpA	FbpA- F	GCACTTGCTATTGCTGCAG	500bp (Pm) 1000bp (Mh)	(36) and This study
	FbpA- R	CTCAGAGAAATCGTCGAA		
TbpA2	TbpA2 -F1	ACGGGATTTGTTTCGCTATG	2300bp (type B)	This study
	TbpA2- F2	TTGGTTGGAAACGGTAAAGC	750bp (A&D), 1300bp (B)	[38] and this study
	•TbpA2- R	ATATGCACTGTCCCACGTCA		
PmSLP	PmSLP- F2	TAGGTTGGGGGCAAGAATG	1400bp (A&D), (1553 (B&E)	[37] and This study
	BRD-PmSLP-F	CCTCACTCGCTCCGACTATT	460bp (PM)	
	HS-PmSLP-F	ATCTGGCAAGTGGGTGATGT	541bp (B& E)	[37]
	•PmSLP-R	TCATCCCAAGTAAAACCCAGTG		
PHSSA	PHSSA-F	TTCACATCTTCATCCTC	327	[23], [29]
	PHSSA-R	TTTTCATCCTCTTCGTC		
Rpt2	Rpt2 -F	GTTTGTAAGATATCCCATTT	1022	[23], [29]
	Rpt2 -R	CGTTTTCCACTTGCGTGA		
sodA	BtsodA-F	GCC TGC GGA CAA ACG TGT TG	144bp	[39]
	Rpt2 -R	CGTTTTCCACTTGCGTGA		

The reverse primers represented diamond sign (•) and written in bold (TbpA2-R and PmSLP-R) in the table were used with more than one forward primer whereas omit this part the amplicon size was determined by estimation for results obtained only in this study.

Table 3: PCR cycling condition for different target genes of *P.multoida, M. hemolytica, and B. trehalosi* isolates

Target gene	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	No. Cycles	Reference
kmt1	95°C/5min	95°C/1 min	55°C/1 min	72°C/1 min	72°C/9 min	30	[33]
capA	95°C/5min	95°C/30sec	55°C/30sec	72°C/30sec	72°C/5min	30	[28]
capB/B:6	95°C/5min	95°C/45 sec	55°C/1min	72°C/1min	72°C/9min	30	[33]
capD/capE	95°C/5min	95°C/30sec	55°C/30sec	72°C/30sec	72°C/5min	30	[28]
FbpA	95°C/5min	95°C/30 sec	50°C/30sec	72°C/1min	72°C/7min	35	[36]
TbpA2	94°C/3min	94°C/30 sec	54°C/30sec	72°C/1min	72°C/10min	30	[35][34]
PmSLP	98°C/30sec	98°C/10sec	60°C/30sec	72°C/1min	72°C/5min	30	[37]
BRD/HS-PmSLP	94°C/2min	94°C/15sec	62°C/30sec	68°C/1min	68°C/5min	35	[37]
PHSSA/Rpt2	95°C/3min	95°C/1min	48°C/1min	72°C/30sec	72°C/5min	35	[23], [29]
Bt-sodA	95°C/5min	95°C/30sec	55°C/30sec	72°C/40sec	72°C/5min	35	[39]

Results

Molecular Capsular Typing of Pasteurella Multocida Isolates

Before running PCR products from all serotypes in a single agarose gel, PCR was conducted separately for P. multocida-specific, type-specific, and HS-causing type-specific genes.

First, PCR performed using P.multocida-specific KMT1 primer produced a unique amplicon size of 460bp for all serotypes. Secondly, PCR was carried out using a type-specific set of primers CapA, CapB, CapD, and CapE separately, and expected PCR products presented at 1044bp, 760bp, 657bp, and 511bp were produced, respectively. Thirdly, HS-causing type B-specific PCR gave an amplification fragment of 620 bp. Finally, the species and type-specific PCR products of the cap gene and HS-causing serotype somatic gene (clone: 6b) were run simultaneously in the same agarose gel.

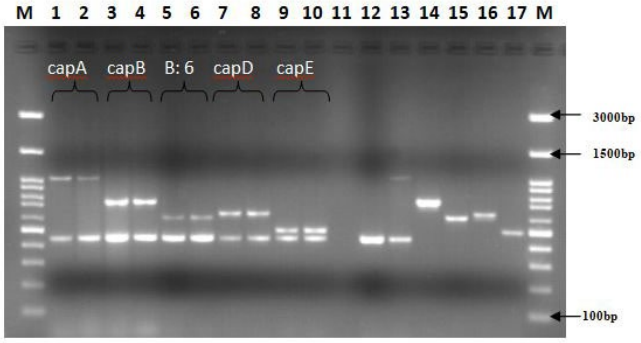

Figure 1: Gel-electrophoresis picture of *P. multocida* specific and type-specific PCR results (two PCR products run for each serotype).

M:100bp DNA ladder M (HiMedia), lanes 1-10 (bottom): PCR product for *P. multocida* specific kmt1 gene (460 bp), lanes 1-2: cap A (1044bp), lanes 3-4: cap B (760bp),lanes 5-6: HS causing type B specific (620bp), lanes 7-8: cap D (657bp), lanes 9-10: cap E (511bp), lane 11: negative control and lanes 12-17: positive controls for *P. multocida* species (kmt1 gene), cap A (with kmt1 gene), capB, B:6, capD & capE, respectively.

Molecular Detection and Genotyping of P.multocida Isolates Based on Virulence-Associated Genes

Detection of Surface Lipoprotein (SLP) Genes of *P. Multocida*

PCR amplification of *P. multocida* surface lipoprotein (PmSLP) gene using PmSLP-Fwd2/PmSLP-Rev primers produced amplicons approximately 1400bp for (A & D) and 1553bp for (B & E) serotypes as indicated Figure 2.

PCR was performed to distinguish bovine respiratory disease and hemorrhagic septicemia causing *P. multocida* variants using forward primers BRD-PmSLP-F and HS-PmSLP-F specific to BRD-PmSLP and HS-PmSLP variants, respectively. The reverse primer (PmSLP Rev) binds to a conserved region outside the PmSLP gene.

PCR amplification band size of 460 bp was detected for both BRD-PmSLP and HS-PmSLP variants using BRD-PmSLP-F/PmSLP-R primer as illustrated in figure 3A whereas HS-PmSLP variants were observed with an amplicon size of 541bp using HS-PmSLP/ PmSLP-Rev primer. BRD-PmSLP variants amplified an additional PCR product at 460 bp as shown in figure 3B for HS-PmSLP/ PmSLP-Rev primer.

We observed that BRD-PmSLP variants (A and D) and HS-PmSLP variants (B and E) were effectively amplified by BRD-PmSLP Fwd/PmSLP-Rev and HS-PmSLP/ PmSLP-Rev primers, respectively.

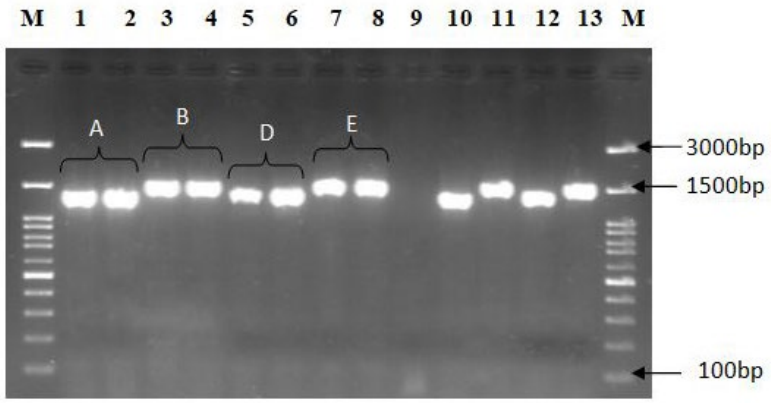

Figure 2: Gel-electrophoresis picture of the PCR amplification of P. multocida surface lipoprotein (PmSLP) gene

M: 100bp DNA ladder (HiMedia), lanes 1-2: P. multocida type A (1400bp), lanes 3-4: type B (1553bp), lanes 5-6: type D (1400bp), lanes 7-8: type E (1553bp), lane 9: negative control, and lanes: 10-13: positive control for A, B, D and E, respectively.

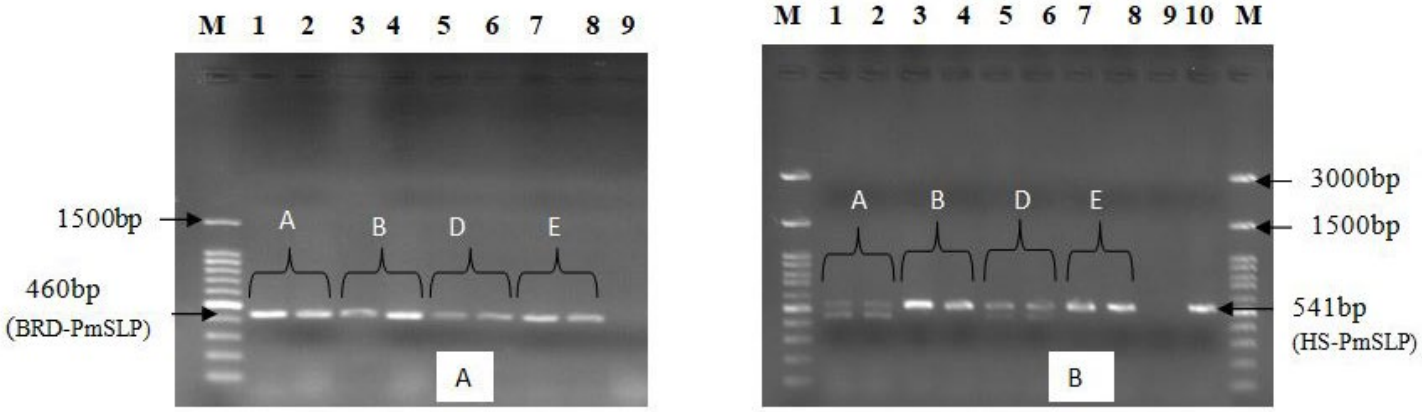

Figure 3: Gel electrophoresis picture of the PCR detection of BRD-PmSLP and HS-PmSLP genes
(A) BRD-PmSLP variant with 460bp PCR product (for all *P. multocida* types) M: 100bp plus DNA ladder (QIAGEN), lanes 1-2: type A, lanes 3-4: type B, lanes 5-6: type D, lanes 7-8: type E, and lane 9: negative control

(B) PCR using HS-PmSLP Primer (460bp &541bp for A & D and 541bp for B& E) M: 100bp DNA ladder (HiMedia), lanes 1-2: type A, lanes 3-4: type B, lanes 5-6: type D, lanes 7-8: type E, lane 9: negative & lane10: positive control

Detection of Iron Acquisition-Related TbpA2 gene in *P. multocida* isolates

The presence of TbpA2 was approved in three capsular serotypes of P. multocida mentioned in Table 1as shown in figure 4. Especially, a large and clear amplification band was detected in the TbpA2 gene of bovine origin type B isolates.

PCR amplification products of approximately 750 bp (type A and D) and 1300bp (type B) were detected using amplification primer TbpA2-F2/Rev. Serotype E was negative for the TbpA2 gene using this primer. PCR product with an amplicon size 2300 bp was produced for only type B serotype using the sequencing TbpA2-F1/Rev primer. However, the other three serotypes (A, D, and E) were not amplified by TbpA2-F1/Rev.

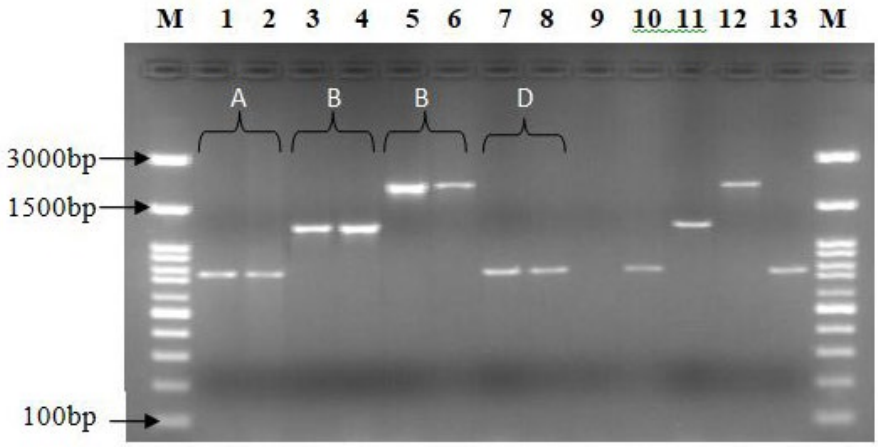

Figure 4: Gel electrophoresis picture of the PCR amplification of TbpA2 gene in *P. multocida* serotypes
M:100bp molecular ladder M (HiMedia), Lanes1-2: *P. multocida* type A (~750bp), Lanes 3-4: P. multocida type B (~1300bp), Lanes 5-6: P. multocida type B (~2300bp using TbpA2-F1/Rev), Lanes 7-8: P. multocida type D (~750bp), Lane 9: Negative control, Lanes: 10- 13 positive control of P. multocida type A, B (using F2& F1primers) and D, respectively.

Detection of iron acquisition FbpA gene in *P. multocida* isolates

All of the *P. multocida* isolates were positive for an iron acquisition gene using P.M FbpA- F/R primer with unique PCR amplicons at 500bp band position visualized on the same agarose gel prepared to run PCR product of FbpA encoding gene for *M. hemolytica* as shown in Figure 6.

Molecular detection of M. hemolytica and B. trehalosi isolates

P.hemolytica serotype-specific-1 antigen (PHSSA) and methyl-transferase (Rpt2) genes of *M. haemolytica* A:1 were detected by using multiplex PCR assay with PCR product size of 327pb and 1022 bp, respectively as indicated in Figure 5. Ferric binding protein (FbpA) gene of *M. hemolytica* (A: 1) was amplified by PCR using M.H FbpA-F/ R primer and identical DNA bands were detected at 1000bp as shown in Figure 6.

Manganese-dependent superoxide dismutase (sodA) gene was amplified using Bt-sodA-F/R primer and PCR amplicon of 144bp produced for all three serovars of *B. trehalosi* (T:3, T:4, and T:15) as illuminated in the following gel picture.

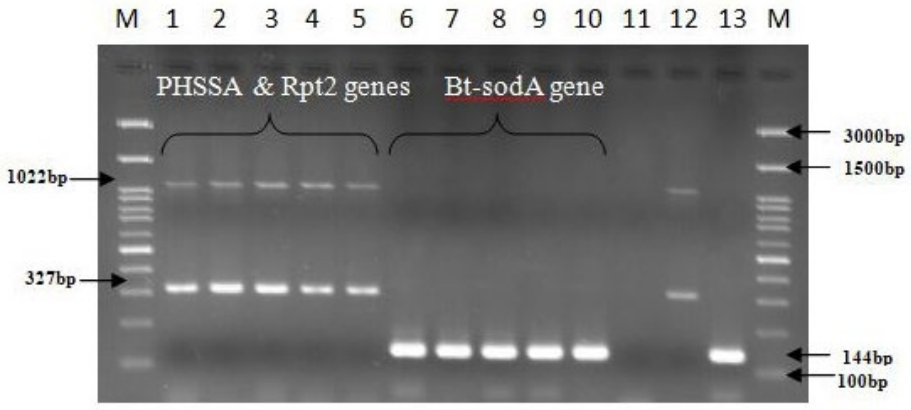

Figure 5: Gel electrophoresis picture showing the PCR detection of PHSSA & Rpt2 encoding genes in *M. hemolytica* and sodA encoding gene in *B. trehalosi* isolates.

M: 100bp DNA ladder M (HiMedia), lane: 1-5 represent *M. hemolytica* A: 1 (327bp for PHSSA & 1022bp for Rpt2 gene), lane: 6-10 *B. trehalosi* (144 bp for sodA gene of *B. trehalosi* isolates T3, T4 and T15), lane: 11 negative control, lane: 12 positive controls for multiplex PCR of *M. hemolytica* and lane: 13 positive control for *B. trehalosi* sodA gene.

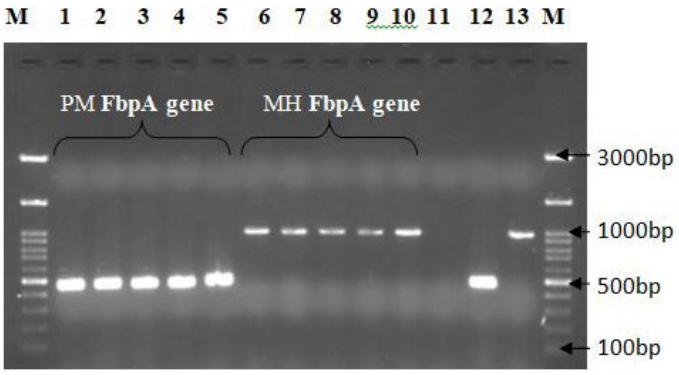

Figure 6: Gel picture showing the detection of ferric binding protein (FbpA) gene of *P. multocida* and *M. hemolytica* isolates.

M: 100bp DNA ladder M (HiMedia), lane: 1-5 *P. multocida* (500bp), lane: 6-10 *M. hemolytica* (1000bp), lane: 11 negative control, lane: 12 positive control for P. multocida, and lane: 13 positive control for M. hemolytica FbpA gene.

Discussion

Many isolates of *Pasteurella*, *Mannhemia*, and *Biberstenia* species are opportunistic pathogens that can cause different animal diseases. They can be isolated from both normal and diseased mammals and birds [1]. Many rapid molecular identification methods have been developed for differentiating *Pasteurella*, *Mannhemia*, and *Biberstenia* isolates [28, 29]. In this study, PCR was performed to detect and genotype based on virulence encoding genes in *P. multocida, M. haemolytica,* and *B. trehalosi* isolates from Ethiopian cattle and sheep at National Veterinary Institute (NVI) as mentioned [32]. *P. multocida*-specific PCR was carried out using primer KMT1T7/KMT1SP6 as a confirmatory test with a unique PCR product of 460bp for all four serotypes (A, B, D, and E). Capsular typing PCR of P. multocida was performed using species-specific set of primers CapA, CapB, CapD, and CapE, and PCR products of 1044bp, 760bp, 657bp, and 511bp were detected for serotypes A, B, D, and E, respectively. PCR amplification was conducted to detect HS-causing serotype B using KTT72/KT SP61 primer produced 620bp amplicon.

All PCR results of this work agreed with previous studies investigated by different researchers except that the authors did multiplex PCR for capsular typing of *P. multocida* serotypes [28, 33]. However in this work, PCR was conducted for each capsular gene of four serotypes (CapA, CapB, CapD, and CapE) and a somatic gene of HS causing serotype (clone 6b) separately. Two PCR products from each capsular type and a somatic gene (clone 6b) were run on the agarose gel at the same time as indicated in figure 1.

The soluble proteins anchored to the outer membrane of Gram-negative bacteria like *P. multocida* are referred to as surface lipoproteins (SLPs). They are displayed by a family of outer membrane proteins called Slam (Surface lipoprotein assembly modulator) and have great roles in immune evasion and nutrient acquisition [3]. All of the Pasteurella serotypes were strong PCR positive for the PmSLP gene using PmSLPFwd2/ PmSLP Rev primer and PCR products approximately 1400bp for type A and D and 1553bp for type B & E were detected. All serotypes of *P. multocida* were also amplified by BRD-PmSLP/PmSLP-Rev primer with PCR product around 460bp as shown in figure 3A. Nevertheless PCR conducted to detect HS-PmSLP variants using a pair of primers (HS-PmSLP/ PmSLP Rev) showed two amplified PCR products (460bp & 541bp) for both A & D serotypes and only a single DNA fragment at 541bp for serotypes of B & E as indicated in Figure 3B. This result indicated that bovine respiratory disease (BRD) causing serotypes A and D produced two fragmented PCR products whereas HS causing types (B & E) yielded a single PCR product using recombinant HS-PmSLP primer. Even though all *P. multocida* serotypes could be amplified by both primers, BRD-PmSLP and HS-PmSLP primers were more popular to amplify BRD and HS causing serotypes, respectively [37].

Most of PCR amplification results obtained from all *P. multocida* serotypes using PmSLP, BRD-PmSLP, and HS- PmSLP genes were agreed with the data recorded in a document provided by Toronto University, Canada. Accordingly, the expected PCR product size of the PmSLP gene is recorded in range as 1553-1589bp for all *P. multocida* serotypes. Even though all the recombinant PmSLPFwd2/PmSLP Rev primers designed focusing hemorrhagic septicemia (HS) - causing *P. multocida* serotypes, we have observed that bovine pneumonic pasteurellosis causing P. multocida serotypes (A&D) were detected by these primers with slightly amplicon size variations. PCR product of HS causing *P. multocida* serotypes (B&E) was found to be approximately 1553bp while bovine respiratory disease (BRD) causing serotypes (A & D) produced approximately 1400bp which was out of the range (1553-1589bp) recorded by Islam for *P. multocida* serotype (B&E) using PmSLPFwd 2/PmSLP Rev. The PmSLP gene amplicon size of HS causing *P. multocida* serotypes (B&E) was somewhat larger than the recorded for BRD-causing serotypes (A & D) as shown in Figure 4.

Based on the protocol developed by the University of Toronto amplification products of multiplex PCR for *P. multocida* serotypes using BRD-PmSLP and HS-PmSLP primers were recorded as 1025bp and 541bp, respectively [37]. But in our study, PCR amplification band at 460 bp was detected for both BRD-PmSLP and HS-PmSLP variants using BRD-PmSLP Fwd/PmSLP Rev primer as illustrated in figure 3A whereas HS-PmSLP variants were observed with an amplicon size of 541bp and BRD-PmSLP variants produced two amplicons at 460 bp and 541bp using HS-PmSLP/ PmSLP-Rev primer as shown figure 3B.

TbpA2 was originally identified in the BRD-causing bovine pathogen, Pasteurella multocida. Recent reports announced that only bovine isolates of P. multocida possess the TbpA2 receptor. TbpA2 receptor of P.multocida binds the N-lobe of bovine transferrin and it has been investigated as a potential vaccine antigen and appears

to offer partial protection against infection [40, 41].

This study showed that single-component receptor transferrin TbpA2 was found to be positive for *P. multocida* serotypes A, B, and D (Figure 4). TbpA2 encoding gene was not detected for serotype E by PCR and this might be due to the lack of transferrin binding receptors in serotype E. We did not evaluate cap F for TbpA2 gene in this work.

The result gained in this research is agreed with the work of who explained that the iron acquisition TbpA gene was found predominant in cap B, followed by cap A and cap D. Other researchers identified the presence of TbpA in four capsular types (A, B, D, and F) with 100% prevalence in F isolates in contrast to the work of Ewers et al. who explained that TbpA was nil in Cap F isolates [34, 42]. PCR products with an amplicon size of approximately ~750 bp for types (A and D) and ~1300bp for type B were found using TbpA2-F2 primers. Similar PCR results released by other researchers using single-component (TbpA2) and bipartite TbpBA-specific primer pairs resulted in the amplification of appropriately sized fragments of ~750bp and ~1300bp, respectively [38].

PCR product with an amplicon size of 2300 bp was produced for only type B serotype using TbpA2-F1/Rev primers. A strong TbpA2 PCR amplification band on the agarose gel was well observed in HS causing serogroup B compared to other capsular types of *P. multocida.*

The ferric binding protein (FbpA) is the nodal point in iron transport from the outer membrane receptor to the inner membrane receptor in certain Gram-negative pathogenic bacteria like *P. multocida.* Due to its function, and the amino acid composition of its iron-binding site, it is the requirement for a synergistic anion to ensure tight iron-binding [43]. After ferric iron ion passed though TbpA pores, it bounds with a second iron binding protein FbpA [40].

PCR was carried out for the detection of an iron-binding protein encoding gene using PmFbpA primer and amplicon size of 500bp was observed for all *P. multocida* serotypes which are consistent with the previous report [36]. The iron acquisition system of *M. hemolytica* (A: 1) allows obtaining iron from the host's iron-binding proteins under iron-deficient conditions [43]. PCR was done for the presence of a periplasmic protein (FbpA) involved in iron acquisition. An amplicon size of 1000bp was detected for all *M. hemolytica* samples.

Mannheimia hemolytica is the primary bacterial pathogen of pneumonic pasteurellosis (shipping fever) in cattle. *Mannheimia hemolytica* biotype A, serotype 1 has been reported to be the most commonly isolated serotype from pneumonic cases [45]. For molecular identification of *M. hemolytica* serotype-specific-1, a multiplex PCR assay was carried out using *P. hemolytica* serotype-specific-1 antigen (PHSSA) and methyltransferase (Rpt2) genes of *M. haemolytica* A:1. PCR amplicon sizes of 327pb and 1022 bp were detected for PHSSA and Rpt2 genes, respectively as indicated in figure 5. This result is in agreement with the previous reports done by different researchers elsewhere [23,29].

B. trehalosi is an important pathogen for young adult sheep, where it causes a well-defined systemic disease [8]. In the present study, three *B. trehalosi* isolates (T3, T4 & T15) were detected by PCR using sodA primer. A small fragment size of amplicon (144bp) was obtained and similar finding with the previous report [29].

Conclusion

Based on the outcomes of this research, various genes expressed in different isolates of *P. multocida, M. hemolytica* and *B. trehalosi* were detected and the distributions of the virulence associated target genes were evaluated using different PCR primers. In addition to the capsular antigens, this study also revealed that SLP, TbpA2, and FbpA were found the major virulence-associated genes in *P.multocida* isolates. Kmt1, SLP and FbpA encoding genes were present in all isolates of P. multocida while TbpA2 was present in only three *P. multocida* isolates (A, B, and D). The virulence gene TbpA2 was predominantly noticed in type B and SLP was dominantly observed in both serotypes B and E.

M. hemolytica (A:1) was found to be positive for serotype-specific and species-specific genes (PHSSA and Rpt2) and iron acquisition gene (FbpA) while the three *B. trehalosi* isolates (T3, T4,&T15) were positive for manganese-dependent superoxide dismutase encoding gene using Bt-sodA primer.

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Availability of Data and Materials

There is no sequencing data generated in this study. However all other data supporting the findings reported can be obtained from corresponding author upon formal request.

Authors' Contributions

G. D. carried out the majority of the laboratory work, data analysis and interpretation of the result, and general manuscript writing, E .G. has significantly contributed to this work in reviewing all the technical aspects., T. A. contributed by full filling all the laboratory facilities to be available in the laboratory and guiding all the activities of the research work., B.G. encouraged the laboratory work and critically revised the manuscript., L.T. collected and isolated all the bacterial isolates and submitted them to the molecular

laboratory with complete information., K.B. was involved directly in laboratory activities and manuscript reviewing. A.L. has participated in some parts of the laboratory work during the research. M.A. contributed to this work by re-isolating some of the bacterial isolates by culturing them in his laboratory. G.Z participated in reviewing the manuscript and advised how it could be organized and prepared for publication. Bo.G. contributed by checking both the technical work of the research and the edition of the manuscript.

Ethical approval

A statement of ethical approval is not applicable, because this this research does not contain any studies with human or animal subjects.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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