RESULTS ON GENETIC ANALYSIS OF *PASTEURELLA MULTOCIDA* ISOLATED FROM LOCAL MONGOLIAN CATTLE

E. Solongo[1], V.Batbaatar[2], B.Enkhtuul[2], Ts.Lundaa[2], J.Khulan[1] and J.Erdenebaatar[2*]

1-School of Art Science, National University of Mongolia, Mongolia
2-Institute of Veterinary Medicine, MULS, Mongolia

[*]-Corresponding author, e-mail: erdene64j@yahoo.com

ABSTRACT

Pasteurella multocida (P.multocida) a small gram-negative coccobacillus, is part of the normal oral flora of many animals, including the pig, bovine, fowl and cat. High morbidity and mortality rates are associated with significant economic losses to the livestock. The aim of this study is to analyze and distinguish phenotypically and genotypically differences between virulent strain isolated from Mongolian cattle and its physically induced mutant strain. For PCR detection, we used specific primers (KMT1T7-FWD, KMT1SP6-R) targeting for kmt1 gene of P.multocida, and specific primers for five serogroups of P.multocida. Nucleotide sequences were analyzed by the Applied Biosystem 3130 xl Genentic Analyzers, using KMT1T7 and KMT1SP6 primers. Completely assembled sequences were aligned with CLUSTAL W and phylogenetic analyses were conducted using MEGA 6.1 version. The results of microbiological testing revealed there is no significant importance discrepancy between strains which are P.multocida 18 and P.mutlocida 144. In fermentation studies, P.mutocida18 were using glucose, sucrose, sorbet, mannose, and mannitol, whereas, it didn't ferment xylose, dulcitol, and raffinose. But P.multocida 144 used fructose, sucrose, sorbet, mannose, mannitol, xylose, and raffinose, whereas it did not use dulcitol. P.multocida detection by standard PCR result was 460bps specific bands and multiplex-PCR showed 750bps specific bands, indicating that 'B' serotype of P.multocida. Phylogenetic analysis showed that Mongolian strains and its mutants were very closely correlated each to other by 93% , while other strains including P.multocida and Pasteurella spp sequences of which were obtained from gene bank were evolutionary related.

KEY WORDS: *Haemorrhagic septicaemia,* kmt1 gene, PCR, partial sequence, virulent strain and mutant strain

INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute and fatal disease caused by *Pasteurella multocida* (*P.multocida*), a gram-negative coccobacillus [1]. This organism usually resides in the mucous membranes of the intestinal, genital, and respiratory tissues and is an opportunistic pathogen that causes cholera in fowl, Bovine Hemorrhagic Septicemia (HS), and porcine atrophic rhinitis. *P.multocida* is a heterogeneous species that produces septicemic or respiratory diseases in domesticated and wild animal

[6]. *P.multocida* strains express a polysaccharide capsule on their cell surfaces and antigenic specificity of the capsule determines the organism's serogroup: A, B, D, E, and F. The major cases of fowl cholera are caused by serogroup A. In pigs, serogroup D are associated with pneumonia and atrophic rhinitis, haemorrhagic septicaemia of cattle, bovine and buffalo is caused by serogroup B and E. Lundaa.Ts and Yondondorj.A (2009) indicated that mainly serogroup A and B overspread in Mongolia. For instance, this study shows that serogroup B had been detected in cattle and serogroup A, and B had detected in flock of sheep [1-3].

However, the underlying genetic relationships of strains representing different serogroups and associated with different host species and diseases is poorly understood. High morbidity and mortality caused by the organism result in significant economic losses to the livestock industry in Mongolia. Conventional methods for diagnosis of *Pasteurellosis* rely on the detection of the causative organism by microscopy and its isolation and identification. Furthermore, it is necessary to develop treatment of *P.multocida* infected animals and increase the efficacy and safety of available vaccines. In this present study, we have investigated the differences between the virulent and its mutant strains. It will be essentially helpful for the further vaccine development.

MATERIALS AND METHODS

Bacteriological study

In this study, we used two strains, which are virulent strain (*P.multocida* 18) and it's physically induced mutant strain (*P.multocida* 144). The strains were grown on a Tryptic Soy Agar (Bacto, USA) at 37°C for 18 hrs. After incubation, a single colony was picked up and prepared a bacterial smear on the glass and stained by Gram staining methods. Bacterial morphology were checked by microscopy.

Biochemical study

P.multocida 18 and *P.multocida* 144 were cultured on Tryptic Soy Broth (TSB-Bacto, USA) and incubated at 37°C for 18 hrs. Fermentation studies were performed using peptone water containing 0.5% sugar and 0.5% acidic fuchsin as an indicator. 100 μl of TSB culture was inoculated 3ml volume of dulcitol or sorbitol peptone water. The cultures were incubated at 37°C and the results recorded after 24 hrs.

Preparation of bacterial DNA

A few colonies were inoculated into Tryptone Soy Agar (TSA-OXOID, England) and grown at 37°C for 18 hrs. After incubation, bacterial culture was harvested and bacterial DNA was extracted by phenol-chloroform method.

Standard Polymerase Chain Reaction (PCR) and Capsular typing via PCR

The sensitivity and specificity of the *P.multocida*-specific PCR offer the most compelling argument for the use of PCR technology in laboratory investigation of suspected HS cases. Standard PCR based on *P.multocida* gene *kmt1* was done to confirm bacterial strains as a P.multocida, as previously described in OIE manual, 2012 [4].

Conditions of standard PCR are as follows: initial denaturation at 95°C for 5 mins, 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, with a final extension at 72°C for 7 mins. Five μl of each sample is electrophoresed on 1.5% agarose gel in 1 X Tris-acetate buffer (TAE) at 100 V/cm for 45 min. Gel is stained with 1% ethidium bromide and DNA fragment are viewed by UV trans-illumination system (Enduro™ GDS, Labnet Inc.) [4].

Detection of capsular genes by PCR for all serogroups was done by according to the method described with some modifications. The oligonucleotide sequence of primers previously published [5].

Multiplex-PCR was used to confirm the strain as *P.multocida* and additionally differentiate between of serogroups A, B, D, E and F. For this multiplex PCR, each reaction mixture (total volume of 25 μl) contained template DNA, 1 X PCR buffer, 200μM each deoxynucleotide triphosphate (dNTP), 2 mM $Mgcl_2$, 3.2 pmol of each primer and 1 u Taq DNA polymerase (Sigma). The used specific primers were: KMT1T7, KMT1SP6 (*P.multocida*) and CAPA-FWD CAPA-REV (1044bps, serogroup A), CAPB-FWD, CAPB-REV (760bps, serogroup B), CAPD-FWD, CAPD-REV (657bps, serogroup D), CAPE-FWD, CAPE-REV (511bps, serogroup E), CAPF-FWD, CAPF-REV (851bps, serogroup F) [5].

PCR condition of initial denaturation at 95°C for 5 min followed by 30 cycle of denaturation (95°C, 30s), annealing (55°C, 30s), extension (72°C, 30s), and final extension step of 72°C for 7 min. Agarose gel electrophoresis is as described above [5]. A positive and negative control (without DNA template) was also included in every run.

Analysis of nucleotide sequences.

Nucleotide sequence were analyzed, edited and assembled with "Applied Biosystem 3130 xl

Genentic Analyzers", using KMT1T7, KMT1SP6 primers. Complete assembled sequences were aligned with CLUSTAL W and phylogenetic analysis were conducted using MEGA 6.1 version. Additionally, we obtained kmt1 gene sequences from NCBI, GENBANK databases with numbers AF016259.1, FJ986389.1, AY225341.1, AY225342.1, AY225343.1, AY225344.1, AY225346.1, AY362916.1, KP036619.1, EU408787.1 [11-20] were used for analysis to our data.

RESULT

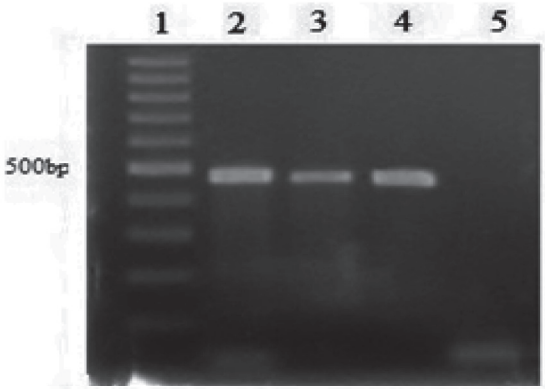
Bacteriologically and biochemicaly tests

P.multocida 18 and *P.multocida* 144 strains are growing with smooth, greyish translucent colonies on TS Agar after 18 hours incubation at 37°C. In the smears both strains were gram-negative, short, ovoid, and like coccobaciili bacterium.

In fermentation studies, *P.multocida* 18 fermented the glucose, sucrose, sorbet, mannose, and mannitol, whereas, didn't ferment xylose, dulcitol, and raffinose. But *P.multocida* 144 fermented fructose, sucrose, sorbet, mannose, mannitol, xylose, and raffinose whereas it did not ferment dulcitol.

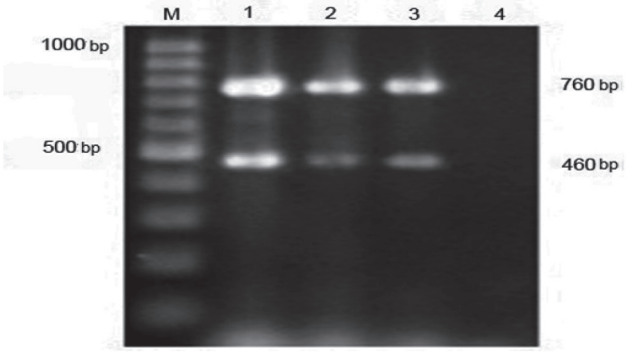
Standard Polymerase Chain Reaction (PCR) and Capsular typing via PCR

By the Standard PCR, both strains were showed approximately 460bps specific bands (*Figure1*)8 which is suggesting that, all strains are *P.multocida.* But, multiplex PCR was successfully optimized and applied to confirm the serogroup of *P.multocida*. *P.multocida* specific primers (KMT1T7, KMT1SP6) are included as an internal control for species identification. The result of multiplex PCR showed 750 bps specific bands indicating that all strains belonged to 'B' serotype of *P.multocida* (*Pic.2*).



Picture 1. The results of *P.multocida* detection by standard PCR.

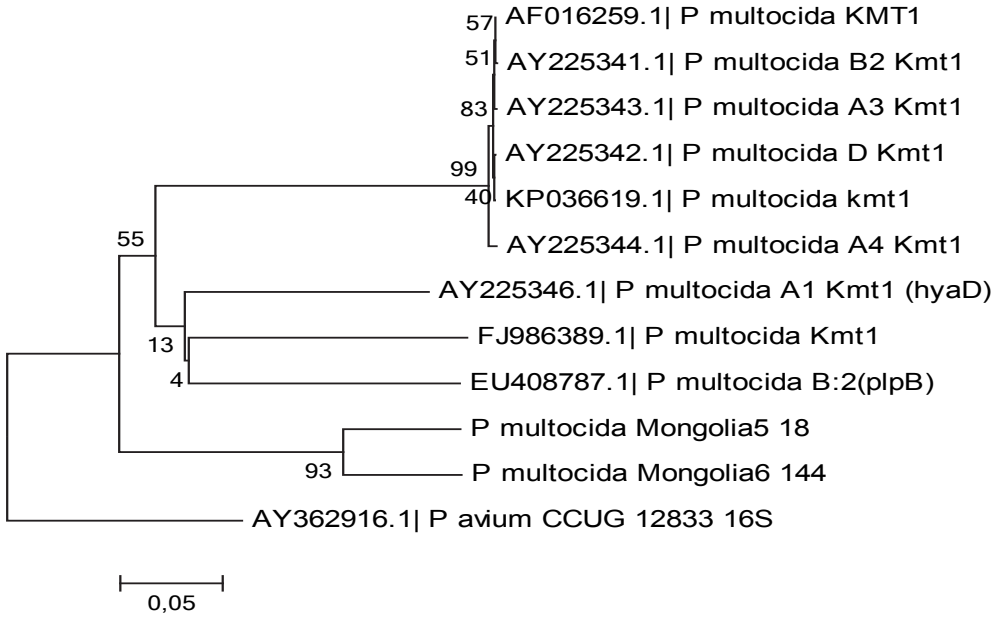
Line 1. 100 bp DNA marker. Line 2. Positive control *of P.multocida*. Line 3. *of P.multocida* 18 (virulent strain). Line 4. *of P.multocida* 144 (mutant strain). Line 5. Negative control.



Picture 2. The results of *P.multocida* serovar detection by multiplex PCR.

Line 1. 100 bp DNA marker. Line 2. Positive control *of P.multocida*. Line 3. *of P.multocida* 18 (virulent strain). Line 4. *of P.multocida* 144 (mutant strain). Line 5. Negative control.

Analysis of nucleotide sequences.
A 1398 bps and 1426 bps fragments of 16 S rRNA that is including the *kmt*1 gene from the virulent and mutant strains was sequenced. The phylogenetic relations of these two *P.multocida strains* obtained in the present study were compared with 10 sequences including *kmt*1 gene sequences of *P.multocida* and 16s rRNA sequence of *P.avium* as shown in Figure 3. Phylogenetic analysis showed that virulent strains and its mutants were very closely correlated to each other by 93%, but other *P.multocida* strains sequences that obtained from gene bank were evolutionary related (Pic.*3*).



Picture 3. Evolutionary relationships of kmt1 gene of *P.multocida.*

The Evolutionary relationship was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [8]. The evolutionary distances were computed using the Maximum Composite Likelihood method [9] and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 355 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [10].

DISCUSSION

Significant morphological difference between *P.multocida* 18 and *P.multocida* 144 strains were not observed, the result was the same as Ts.Lundaa, A.Yondondorj (2002, 2009). Ts.Lundaa et al (2002, 2009) and manual of OIE, 2012 reported that due to the biochemical activity of this organism glucose, sucrose, sorbet, mannitol, sorbitol galactose were fermented not producing gas whereas the organism didn't ferment lactose, dulcitol, and raffinose. *P.multocida* 18 strain isolated from local Mongolian cattle fermented carbohydrates as mentioned above but it's mutants *P.multocida* 144 additionally fermented lactose, and raffinose whereas it didn't dulcitol. Standard PCR based on kmt1 gene proved that it is *P.multocida.* In multiplex-PCR result, we have obtained serogroups 'B' by showing specific bands 760bps and 460 bps as a previously published result of researchers Arumugam and Blackall of Malaysia and Australia [3].

Partial sequencing result was used to build a phylogenetic tree showed that Mongolian strains and its mutants were very closely correlated and it indicates more evolutionary relation than other *P.multocida* strains. Genetically, 93% were similar to each other, and *P.multocida* 18 and *P.multocida* 144 strains that identified as serotype "B" having different biochemical activity need be further studied

CONCLUSION

1. In the morphological term, there is no significant importance discrepancy between these strains *P.multocida* 18 and *P.multocida* 144.
2. Confirmation of both virulent and mutant strains using standard and multiplex PCR demonstrated it is *P.multocida* and its serotype "B".
3. The characteristics of these two strains are evolutionary correlated each to other by 93% as compared with randomly selected 10 different strains of *P.multocida* and other *Pasteurella spp*.

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