Role of Molecular Techniques in Characterization of Bacteria Causing Pneumonia in Small Ruminants

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Abstract: Sheep and goats are important agricultural animals in many developing countries which can compensate the shortage in cattle and buffaloes meat production besides wool and fiber. The most important reason of the sheep and goats losses is pneumonia in a percentage of 24-51% which causes economic losses from fatal pulmonary infections and impairment animal performance. Pneumonia could be occurred due to many fastidious bacterial pathogens, therefore the conventional methods for isolation and identification of these bacteria are useless. Nucleic acid-based assays have been established as gold standard for precise molecular identification and phylogenetic relationship within the family as well as on subspecies level. For instance; the 16S rDNA gene sequencing has been revealed that many isolates previously classified as ''*Mannheimia haemolytica*'' were *Mannheimia glucosidal* or *Mannheimia ruminalis*. Genomic subtraction studies identify a unique chromosomal region has successfully been used to detect *Pasteurella multocida* from suspected samples. Phylogenetic relationships of representative sheep and goat *P. multocida* serotype B: 2 isolates revealed two polymorphic sites were present in goat isolate *vs* one in sheep isolate. Polymerase chain reactions (PCRs) can be reliably used on mycoplasmas growing in culture even in the presence of bacterial contamination. The restriction enzyme analysis of *rpo*B gene may be useful to differentiate ovine strains from bovine strains of *Histophilus somni*, 16S rDNA gene amplification of *Helcococcus ovis* strain isolated from goat revealed that the nucleotide sequence matched 96%identity with that isolated from sheep. The molecular characterization can be used for early detection and precise discrimination of fastidious pathogens phylogeny as well as virulence genes as a future step for vaccine preparation.
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Introduction

Sheep and goats represent an important standing in the domestic animal wealth especially in Middle East and Arabic countries. They could be compensate the shortage of milk and meat supplies' of cattle and buffaloes .Besides they kept as a source of wool, fiber and hide ***(Baker and Grey, 2004)***. In the same time sheep husbandry plays a multifaceted role of the rural households by providing mutton, wool, manure and hides ***(Latief et al., 2013)***. The goat is an important commodity in many areas of the world, where it is kept as a source of meat, milk and fiber. Often described as the "poor man's cow," the goat can survive in areas where a cow cannot ***(Clothier et al., 2012)***. In Egypt, sheep and goat numbers were estimated to be about 3.5 and 3.3 million head, respectively. Sheep represent an important member of the livestock and are kept mainly for meat and wool production, where they yield about 10.4% of meat consumption; therefore, a great attention should be directed toward caprine and ovine industry to meet the people animal protein requirements ***(Dardeer et al. 2006)***. Respiratory illnesses are the major cause of deaths in the lambs and kids besides decreased productivity in the older animals. The most common problem associated with the lower respiratory tract is pneumonia ***(Ramirez- Romero and Brogden, 2000)***. Pneumonia could be occurred due to bacterial pathogens, the most common bacterial species involved are *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma ovipneumoniae* spp. The former bacteria are fastidious in their growth onto culture media and posses complex antigenic structures, therefore the conventional methods for isolation and identification are useless ***(Safaee et al., 2006)***. Genotypic characterization has proved beneficial in overcoming limitations of traditional phenotypic procedures, facilitates identification, improves the sensitivity and allows the direct detection of organisms from clinical samples ***(Hunt et al., 2000)***. In the present review, the role of molecular tools in characterization and discrimination of bacteria causing pneumonia in small ruminants will be discussed.

I. Phylogeny tree or reclassification and nomenclature of bacteria

Phylogenetic comparison of universal bacterial sequences (16S rDNA), gene sequences has recently

become a key character for bacterial classification. This has resulted in improved classifications of many groups and enabled re-evaluation of the selection of phenotypic characters used for identification. Comparison of 16S rDNA gene sequences might also be used directly for identification or result in subsequent development of polymerase chain reaction tests targeting specific regions of the 16S rDNA genes, ***(Christensen et al., 2003a).***

Formerly genus *Pasteurella* constitutes two species *Mannheimia* haemolytica (*M. haemolytica*) and *Pasteurella multocida* (*P. multocida*), *M. haemolytica* is a heterogeneous bacterial pathogen as there are two biotypes have been recognized; biotype A consisting of isolates that ferment L-arabinose and biotype T consisting of isolates that ferment trehalose. Together they are represented by 1–17 M. haemolytica serovars. Recently these recognized serovars represent three genetically distinct species. Serovars A1, A2, A5 to A9, A11 to A14, A16, and A17 represent *M. haemolytica* and serovars 3, 4, 10 and 15 represent *P. trehalosi* while serovar 11 represents *Mannheimia glucosida* ***(Miller, 2001)***.

Comparison of 16S rDNA sequences of *M. haemolytica* isolates has shown that the A1, A2, and A11 serotypes represent distinct phylogenetic lineages ***(Highlander et al., 2001)***. The multiplex PCR of primer sets included hypothetical protein (HP), "amplifying a DNA region from an unknown", *lkt* , *lkt*2, amplifying different regions of the leukotoxin D gene and amplification of the 16S rDNA gene. Based on positive amplification, isolates were delineated as *M. haemolytica* (HP, *lkt*, 16S), *M. glucosida* (HP, *lkt, lkt2*, 16S), or *M. ruminalis* (HP, 16S) ***(Alexander et al., 2008)***. Phylogenetic analyses revealed that many isolates previously classified as ''*M. haemolytica*'' were *M. glucosidal* or *M. ruminalis*. ***(Miller et al., 2013)***.

Pasteurella trehalosi are well-known pathogens of ruminant world-wide, formerly, belonged to *P. haemolytica* species, as it could ferment trehalose ***(Odugbo et al., 2004)***. *Pasteurella trehalosi* is closely related to *Mannheimia* phenotypically as well as in the 16S rDNA tree. However, the *rpo*B tree (*rpo*B is the gene that encodes the β subunit of bacterial RNA polymerase) separates this species from the *Mannheimia* cluster ***(Korczak et al., 2004)***. Analysis of genomic DNA of each *M. haemolytica* (35ovine and 21caprine) and *P. trehalosi* (19 ovine and 2caprine) isolates by PFGE showed a higher level of discrimination as many unique PFGE patterns were observed and the molecular patterns of *P. trehalosi* T3, T4 strains were typically different from those isolates which belong to *M. haemolytica*; no common PFGE pattern was found between isolates of both species ***(Villard et al., 2006)***.

Amplified fragment length polymorphism (AFLP) was performed on the *P. trehalosi* type strain NCTC 10370T, field isolates from sheep (21) and goats (1). All *P. trehalosi* isolates shared at least 70% 16S rDNA gene sequence similarity in AFLP patterns. The largest AFLP patterns were the type strain and 7 ovine field isolates with 98.6% or higher. In addition, only 62% or less DNA–DNA relatedness has been found between *P. trehalosi* and other members of the family Pasteurellaceae proposing the transfer of P. trehalosi to a new genus; *Bibersteinia*, as *Bibersteinia trehalosi* (*B. trehalosi*) comb. *nov*. ***(Blackall et al., 2007)***.

Phylogenetic analyses and *lkt*A PCR data provided insights into relationships among the 25 trehalose-fermenting *Pasteurellaceae* isolates obtained from bighorn sheep (BHS). Sixteen of those isolates grouped with *B. trehalosi* reference strains but only nine are *lkt*A positive and appeared to be closely related. Based on 16S rDNA sequencing, these nine trehalose-fermenting isolates formed a clan divergent from B. trehalosi reference strains. Sequence similarities between these nine isolates and the three reference strains ranged from 95.3% to 98% ***(Miller et al., 2013)***.

P. multocida is classified into three subspecies based on DNA–DNA hybridization, namely *P. multocida* subsp. *gallicida*, *P. multocida* subsp. *multocida* and *P. multocida* subsp. septic, the DNA–DNA hybridization study indicated that the three subspecies could be classified as distinct species **(***Blackall et al.1998)*, however, more recent data based on ribotyping indicate that the three *P. multocida* subspecies do not represent distinct genotypic groups ***(Muhairwa et al., 2001; Petersen et al., 2001).***

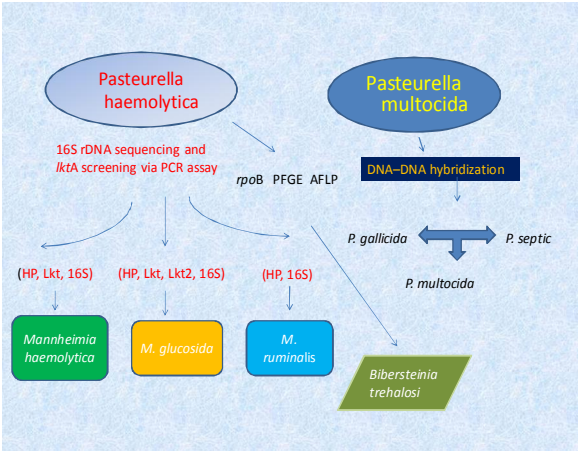

Figure 1: Recent phylogeny tree, reclassification and nomenclature of genus *Pasteurella*

Another DNA–DNA hybridization studies, 16S ribosomal DNA gene and *rpo*B sequencing reclassified genera previously referred to as *Haemophilus somnus* (*H. somnus*), *Histophilus agni* (*H. agni*) and *Histophilus ovis* (*H. ovis*) to a new sole species *Histophilus somni* ***(Angen et al., 2003***; ***Dousse et al., 2008)***.

II. Inter and intra-species Discrimination

PCR assays are beneficial in differential diagnosis of *Mycoplasma agalactiae* and *Mycoplasma bovis* which antigenically shared. The advent of diagnostic molecular tests, especially real-time PCR has provided rapid, sensitive and robust tests for identifying mycoplasmas ***(Oravcová et al., 2009***; ***Becker et al., 2012)***.

The phylogenetic tree analysis of the *rpo*B gene showed that the ovine strains appeared to form a subgroup recovered in 70% of the bootstrap trees. In the 311 bp region of the ovine strains, a *Hinc*II restriction endonuclease site was detected. The PCR-amplified *rpo*B DNA of 46 bovine and 20 ovine *H. somni* strains were examined for the digestion with *Hinc*II. As the results, 17 strains of ovine strains were cleaved by the enzyme appeared to possess the restriction site. The restriction enzyme analysis of *rpo*B gene may be useful to differentiate ovine strains from bovine strains of *H. somni* ***(Tanaka et al., 2005)***.

Sequencing of SC01complete genome (a *M. ovipneumoniae* strain isolated from the lung of a goat with pneumonia in Sichuan Province, China) revealed highest percentage of initiation codon among available mycoplasma genomes ***(Yang et al., 2011)***.

Comparative sequence analysis of the 16S rDNA gene of *P. multocida* serotype B: 2, the sheep isolate (PM82) shared 99.9% homology with (HS vaccine strain, P52) showing one polymorphic site, whereas goat isolate (PM86) shared 99.8% homology revealed two polymorphic sites ***(Dey et al., 2007).***

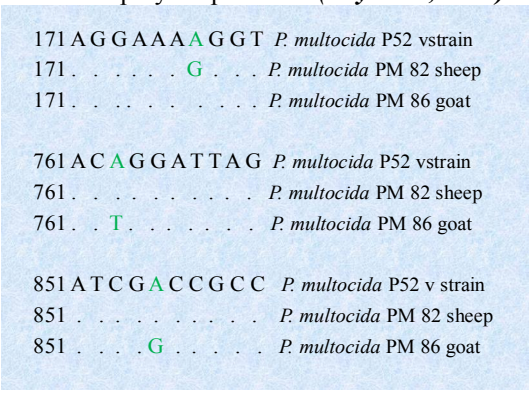

Figure 2: Comparative sequence analysis of *P. multocida* serotype B: 2: sheep isolate, goat isolate with HS vaccine strain, P52.

III. Diagnosis of Fastidious Microorganisms

Conventional microbiological methods can fail to isolate mycoplasmas, ***(Besser et al., 2012)***. Several PCRs specific for mycoplasmas based on different gene sequences have been developed and reported to be more sensitive than culture. They can be used directly on secretory and tissue samples as *M. ovipneumoniae* was detected in the bronchoalveolar lavage fluid from pneumonic BHS lambs by 16S rDNA species-specific PCR sequences ***(Dassanayake et al., 2010)***. PCR greatly facilitates the detection of mycoplasma growing in a 24 hour enrichment culture even in the presence of bacterial contamination ***(Weiser et al., 2012)***. *Mycoplasma capricolum* subsp. *capripneumoniae* (MccF38) is very difficult to grow in vitro, as PCR, which can be carried out directly on the pleural fluid or affected lung, has greatly facilitated the diagnosis of CCPP. Individual PCRs have been reported for *Mcc* ***(Dassanayake et al., 2010)***.

IV. Precise diagnosis

Genomic subtraction studies enabled identification of a unique chromosomal region, a 460 bp product from all *P. multocida* strains; successfully has been used to detect *P. multocida* from suspected samples ***(Townsend et al., 2000)***. A PCR test based on two putative transcriptional regulators (Pm0762 and Pm1231) gave products of 567 bp and 601 bp respectively, these genes appeared to be unique and have recently been described for the detection of *P. multocida* ***(Liu et al., 2004)***. The *pls* gene encodes for a protein that is unique to *P. multocida* and *Haemophilus influenzae* and a positive sample gives an amplicon of 453 bp in size ***(Dziva et al., 2008)***.

The *Mannheimia*'s *sod*A sequences showed a markedly higher divergence than the corresponding 16S rDNA genes, confirming that *sod*A is a potent target to differentiate related species. No cross reactivity was observed, indicating that the assay accurately identified the examined *Mannheimia* spp. ***(Guenther et al., 2008)***.

V. Characterization of virulence genes

Identification of the important virulence factors of an organism is a pre-requisite for understanding the mechanism(s) of pathogenesis and developing control measures ***(Hodgson et al., 2003).*** *M. haemolytica* produces several virulence determinants, of which leukotoxin (Lkt) and lipopolysaccharides (LPS) are considered to be the most important ones and has been accepted as the major virulence factors ***(Jeyaseelan et al., 2002***; ***Subramaniam et al., 2011).*** Leukotoxin (*Lkt*) secreted by *M. haemolytica* is responsible for the pathogenesis of pneumonia. *Lkt* is

a 102 kDa protein belonging to the repeats in toxin (RTX) family of pore-forming toxins; at high concentrations the toxin induces trans-membrane pore formation, colloidal osmotic swelling, and eventual cytolysis ***(Lawrence et al., 2008)***. ***Vougidou et al. (2013)*** investigated *lkt*A sequence variation among ovine and caprine *M. haemolytica* strains isolated from pneumonic lungs. Data analysis showed that the prevalent caprine genotype differed at a single non-synonymous site from a previously described uncommon bovine allele, whereas the ovine sequences represented new, distinct alleles.

PCR-based method was used to amplify the RTX domain of the *Pasteurella lkt*A gene which was detected in 108 (44%) isolates ***(Ewers et al., 2006)***.

It was suggested for epidemiological studies in *P. multocida* on the basis of what is called virulence genotyping and facilitated the examination for capsular and many virulence associated genes. The virulence associated genes (*tbp*A, *pfh*A, *tox*A, *hgb*B, *hgb*A, *nan*H, *nan*B, *sod*A, *sod*C, *oma*87 and *ptf*A) play important role in pathogenesis of *P. multocida* ***(Ewers et al., 2006)***. Among these previous important virulence genes, two were related to iron metabolism *tbp*A and *hgb*B have been identified in pathogenesis of *P. multocida*, while *phf*A and *tox*A have different roles in bacterial virulence. The former has a role in bacterial adhesion and the latter is responsible for producing dermo-necrotoxin ***(Shayegh et al., 2008)***. Multiplex PCR techniques for *pfh*A, *hgb*B, *tbp*A and *tox*A genes revealed that homologous genes were identified in most types A, D, and F isolates and improved methods for laboratory typing of *P. multocida* isolates ***(Townsend et al., 2001)***.

Analysis of serovars 1 and 14 revealed that the LPS produced by each strain are structurally distinct; however, sequence analysis of their LPS outer core biosynthesis' loci revealed that they both contained the same set of genes which clustered within a single locus, between the conserved genes *fpg* and *pri*A ***(St Michael et al., 2005***; ***Harper et al., 2007b***; ***Boyce et al., 2009*** and ***Harper et al., 2011).***

In contrast, the analysis of the *P. multocida* genome revealed that the genes required for the assembly of the inner core of the LPS (*kdt*A, *kdk*A, *hpt*A, *hpt*B, *hpt*C, *hpt*D, *lpt*-3, *gct*A and *gct*B) are located in several regions of the genome; *kdt*A, *kdk*A, *hpt*A, and *gct*B are located within the one locus, while the genes for *hpt*B and *hpt*C are encoded together elsewhere on the genome ***(Harper et al., 2007a)*** and the three remaining transferase genes (encoding *hpt*D, *gct*A and *lpt*-3) are each located separately on the *P. multocida* genome ***(St Michael et al., 2009)***.

M. ovipneumoniae strain SC01 contains several recognizable genes likely to be involved in virulence. Two genes encode proteins are highly similar to bacterial toxins, hemolysin A (*hly*A) and hemolysin C (*hly*C). ***(Calderon-Copete et al., 2009)***.

H. somni Lipooligosaccharide (LOS) is an endotoxin, which can undergo phase variation in composition and structure in vitro or in response to a mounting immune response by the host ***(Corbeil, 2007)***.

Future sight

Despite extensive research activities including the genome analysis lead to the identification of several new potentially virulence associated genes, there are a lot of open questions concerning the molecular pathogenic mechanisms of the bacterial species. Problems encountered are the high antigenic variability and the wide host spectrum as in case of *P. multocida* as well as different courses of infection which also imply enormous difficulties in producing vaccines. An alternative approach to control pathway could be the development of disease specific subunit vaccine depending on bacterial virulence genes sequencing. During the comparative genomic sequence analysis of the *M. haemolytica* isolates a number of unique genes were identified. These genes are "high value targets" for future studies that attempt to correlate the variable gene pool with phenotype. Also a number of high confidence single nucleotide polymorphisms (hcSNPs) were identified and spread throughout the genome. These SNPs will be used to design new hcSNP arrays to study variation across strains, and will potentially aid in understanding gene regulation and the mode of action of various virulence factors.

Conclusion

Respiratory affections of small ruminant especially Pneumonia are regarded as a frequent cause of economic losses in lambs and kids and its etiology involves many different factors including bacteria such as members of family *Pasteurellacaea* and mycoplasmals. These bacteria are fastidious organisms and complex in their antigenicity, therefore the conventional methods for identification are not valuable needing to more recent and discriminating techniques which include various DNA and RNA – dependent techniques.

The advent of diagnostic molecular tests have been applied; provided rapid, sensitive and powerful tests for identifying and discriminating these fastidious bacterial species.

DNA sequence–based identification and comparative sequence analysis of the 16S ribosomal DNA (rDNA) coding gene as well as certain housekeeping genes have been successfully used for identification and for clarifying the phylogenetic

relationship within the family as well as on subspecies level.

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