MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF *MYCOPLASMA SYNOVIAE* ISOLATED FROM CHICKEN

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ABSTRACT

Mycoplasma synoviae is one of the paramount causes of economic losses for poultry industry. Present study was carried out with the aim to isolate, identify and characterize *M. synoviae* from suspected chicken. Tracheal and cloacal swabs samples (n=500), collected from suspected chicken, were cultured on Modified Frey, s medium for isolation of *Mycoplasma*. Preliminary identification of *Mycoplasma* was done by culturing and biochemical characterization. Partial 16 S r RNA was amplified and sequenced for confirmation and molecular identification of *M. synoviae*,. For strain identification and phylogenetic analysis, partial amplification and sequence of vlhA gene was performed. Out of 500 samples, *Mycoplasma* was successfully isolated from 40 samples. Out of 40 mycoplasma isolates, 20 were identified as *M. synoviae* by specie specific 16S ribosomal RNA gene, partial amplification (~213 bp). The vlhA gene was also successfully partially amplified (~375 bp) and sequenced from five isolates. GenBank accession numbers of reported local isolates in this study are KJ130523-KJ130542.and KP316017-KP316021 respectively.

Key words: Mycoplasma, fried egg colonies, 16S rRNA, vlhA gene.

INTRODUCTION

Mycoplasma is one of the major respiratory tract pathogen of chicken, causing considerable losses to poultry industry (Dufour-Gesbert *et al.,* 2006). *Mycoplasma synoviae* and *Mycoplasma gallisepticum* are considered as most pathogenic avian strains (Yilmaz *et al.* (2011). *M. synoviae mostly* infects upper respiratory tract as a subclinical infection but if It combines with *M. gallisepticum* it may causes air sac lesions (Silva *et al.,* 2008). *M. synoviae* causes heavy losses due to reduction in egg production and growth rate, loss of egg production, egg shell abnormalities, low hatchability and lame birds culling (Vogel *et al.,* 2008; Peebles *et al.,* 2011). *M. synoviae* transmission occurs both vertically and horizontally. *M. synoviae* is transferred through infectious aerosols which are generated by coughing and sneezing of infected birds, contaminated feed, water, feather, personal contact and direct contact of susceptible birds with infected chickens (Morois *et al*., 2005; Butcher and Jacob, 2009). Immune suppression in birds enhances the spread of infection (Dhondt *et al.* (2007). Tried and true diagnosis is required to prevent infection dissemination (Seifi and Shirzad, 2012).

For the screening of *M. synoviae* in chicken flocks rapid slide agglutination test (RSA) and Enzyme linked immunosorbent assay (ELISA) are widely used. Species-specific 16S ribosomal RNA gene based PCR is also used to detect the infection but due to conserved nature of gene it does not allow strain differentiation of *M. synoviae.* Although there is only one serotype of *M. synoviae* present but heterogeneity among *M. synoviae* strains is observed (Kleven, 2003) which can be determined by amplification and sequencing the vlhA gene. *M. synoviae* vlhA gene is expressed as two products including hemagglutinin and prolipoprotein (Noormohammadi *et al.* (1998). The hemagglutinin has high frequency of antigenic and phase variation **(**Noormohammadi *et al.,* 2000; Kleven, 2003). Recombination of vlhA gene at 3´ end with one of the pseudo genes can produce antigenic variants which may lead to evasion of host immune system. Although vlhA gene 5´end is relatively conserved and present as a single copy, but pseudo genes can replace its downstream region (Noormohammadi *et al.,* 2000). For the isolation and control of prevalent *M. synoviae* it is very important to characterize its isolates up to its strain level for better understanding. For PCR based strain identification the upstream region of 5´of vlhA gene is used in outbreak and epidemiological studies (Hong *et al.,* 2004; Hammond *et al.,* 2009).

Although there are many studies throughout the world which report the isolation and molecular characterization of *M. synoviae* from chicken, only a few reports are available from Pakistan. Therefore, present study was designed for isolation, and identification of *M. synoviae* from dubious chicken, partial amplification of vlhA and 16S rRNA gene and sequencing of amplicons

for phylogenetic analysis to determine the heterogeneity of M. synoviae isolates.

MATERIALS AND METHODS

A total of 500 samples including tracheal/cloacal swabs (n=250) and morbid materials such as joints, trachea and lungs (n=250) were collected from chicken suspected with *Mycoplasma* infection. Trachea and cloacal cleft swabs were transported in Aimes transport media to University Diagnostic Laboratory (UDL), University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. Morbid materials including trachea, air sacs, lungs and joints were collected and transported to lab in polythene bags with proper labels and case history.

Isolation of Mycoplasma: For the isolation of Mycoplasma, all samples were cultured in Frey' s media (Lavric *et al.,* 2007). Each set of five samples were cultured as a unit. Tracheal/cloacal swabs were cultured directly. Joints were opened, swabbed from internal surface of joint cavity and cultured. Other organs such as trachea and lungs were grinded with phosphate buffer saline, centrifuged at 1000 rpm for 20 minutes and supernatents were filtered through a syringe filter (0.22µm) and cultured at 37 ºC in 5% carbon dioxide. Cultures were observed for any change in color and turbidity. Initial cultures were filtered with or without showing any growth through 0.22µm filter and sub cultured in modified Frey' s broth and agar. Cultures were observed daily for 21 days for any change in color, turbidity and presence of typical fried egg shaped colonies on agar plates, to be declared as negative.

Biochemical identification: Culture was identified as mycoplasma by biochemical characterization and microscopic examination. For microscopic examination the impression smears were prepared from fried egg colonies, stained by Giemsa stain and observed at 1000X magnification using bright field microscope. The biochemical tests performed for suspected cultures were glucose fermentation, arginine hydrolysis and tetrazolium dye reduction test (Ehtisham *et al.,* 2011).

Molecular characterization: The isolates, identified as Mycoplasma on the basis of morphological, cultural and biochemical characteristics, were confirmed as *M. synoviae* by specie specific 16S r DNA PCR. Briefly, DNAs of all isolates were extracted by QAIGEN DNA extraction kit. Partial 16S DNA was amplified by using *M. synoviae* specific primer pair MS-F; 5'-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3' and MS-R: 5'CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-3' (Lauerman, 1998). Reaction mixture (25 µL) had ~20 n mol of genomic DNA and 25 p moles of reverse and forward primer. In Amplification cycle Initial denaturation occurs at 95 ºC for 5 minutes, denaturation at 95 °C, 35 cycles of 30 second each, , annealing at 49°C, one minute extension at 72 ºC , and final extension at 72°C for 5 minutes was done. Amplicons (213 bp) were resolved on agarose gel (2 %), stained with ethidium bromide (0.05 µg/ml) and visualized in UV illuminator and gel documentation system (Bio-Rad, USA)

Partial amplification of N-terminal of vlhA gene: MS vlha- F; 5'-GGC-CAT-TGC-TCC-TAC-TGT-TAT- 3' MS vlha- R; 5'-AGT-AAC-CGA-TCC-GCT-TAA-TGC-3' following Wetzel *et al*., (2010) were used to amplify vlhA gene N-terminal using 56° C annealing temperatures. Amplicons (375bp) were resolved on agarose gel (2%), stained with ethidium bromide (0.05 µg/ml) and visualized in UV illuminator and gel documentation system (Bio-Rad, USA)

Sequencing and Phylogenetic analysis: Partial 16S r DNA amplicons (213 bp) and vlhA amplicons (385 bp) were sequenced by Sanger method. Sequence results were analyzed using Bio Edit software and sequence homologies were determined by Clustal W. Gen Bank accession numbers for all the sequences were obtained from NCBI. Phylogenetic trees were constructed by MEGA 6 version, using NJ method with 1000 bootstrap value.

RESULTS

Total of 500 samples were cultured as 100 units (each unit contained five samples). Out of 100 units, growth of *Mycoplasma* was observed from 40 units (40%) including 20 units of tracheal /cloacal swabs, 15 units of joints and 05 units of samples of trachea, lungs and air sacs. Growth of *Mycoplasma* was observed as typical egg fried shaped colonies on Frey´s agar (Fig. 1a). Impression smears prepared from egg fried colonies were stained with Giemsa' s stain and *Mycoplasma* specific pleomorphic coccoid bodies were observed under microscope at 1000X magnification (Fig. 1b). All isolates (n=40, MYC01-MYC40) were positive for glucose fermentation while negative for arginine hydrolysis. Isolates showed variable results for tetrazolium reduction. Out of 40 Mycoplasma isolates, 20 isolates showed *M. synoviae* specific amplification (amplicon size ~213 bp) of 16S rDNA by PCR (Fig 2). Analysis of partial sequences also revealed that all these 20 isolates were *M. synoviae*. GenBank Accession numbers of these isolates are KJ130523-KJ130542. Phylogenetic tree of *M. synoviae* isolates (n=20), on partial 16S r DNA sequences, revealed that all isolates had close genetic relatedness (> 99% similarity (Fig. 3). Furthermore, phylogenetic tree based on 16 S r DNA (Partial amplification) also revealed that *M. synoviae* isolated in this study had close relatedness with isolates from Iran, India and Egypt. The vlhA gene was successfully amplified (figure 04) from 05 isolates. Sequences of vlhA

gene (Partial amplification) were submitted to NCBI. GenBank accession numbers are KP316017-KP316021. Phylogenetic tree, constructed on the basis of sequences of vlhA gene amplicons (partial), revealed that isolates had similarities with isolates from Europe and USA (Fig. 5).

DISCUSSION

Mycoplasma synoviae is considered as one of the important leading agent to be responsible for economic losses in poultry sector. Poultry flocks are generally screened for *M. synoviae* by rapid slide agglutination test, enzyme linked immunosorbent assay and polymerase chain reaction throughout the world (Hess *et al.,* 2007). Isolation of *M. synoviae* is considered as touchstone for confirmation of infection. Isolation of *M. synoviae* is a prerequisite for its molecular characterization, phylogenetic analysis and possible source tracking (Bayatzadch *et al.,* 2011; Buim *et al.,* 2009). Isolation of *M. synoviae* is not preferred for screening of large flocks because of difficulties in its culture and low recovery rate. Phenotypic and genotypic characterization of *M. synoviae* helps in future diagnostic and epidemiological studies (Moreira *et al.,* 2015).

M. synoviae being a fastidious organism tends to disappear from the lesions after few weeks so its isolation and recovery rate is dependent on the time and site of sampling. In present study different types of samples including tracheal/cloacal cleft swab, trachea, air sacs, lungs and joint were collected to enhance the recovery rate of *M. synoviae* (Anonymous, 2008; Hennigan *et al*., 2012; Ehtisham *et al*., 2011; Pourbakhsh *et al*., 2010). *M. synoviae* were successfully isolated on Frey's media (pH 7.8) at 37 º C in 10% CO_2 and high humidity. Similar growth conditions have been used previously by Tebyanian *et al.,* 2014). Cultured samples, with or without any indication of growth, were sub cultured in fresh Frey's medium after filtering through 0.22 µM syringe filters. Previous studies have used filtration of sample or initial culture through filter paper of 0.45µM pore size to remove the contaminants (Bayatzadch *et al.,* 2011; Tebyanian *et al.,* 2014). In present study, filtration was done through a filter paper of 0.2 µM pore size, which proved to be more efficient for the isolation and purification of *M. synoviae*. Growth of *mycoplasma* was observed by change of phenol indicator in Frey's media from red to orange and yellow as mycoplasma produces acid metabolites and lowers the pH of media. Low pH is injurious for mycoplasma survival, so it is very important to remove the positive samples from incubation and kept under refrigeration for further processing. Overall recovery rate of Mycoplasma (40%), reported in this study, is in meeting of minds with previous studies (Benicina *et al.,* 2001, 2005; Lavric *et al.,* 2007; Nikfarjam and Farzaneh, 2012). *Mycoplasma* showed fried egg shaped colonies on solid media (Kleven, 2003; Kleven and Ferguson-Noel, 2008; Ehtisham *et al.,* 2011).

Although 16S rDNA is the most conserved region in bacterial genome, it still contains specie specific signature sequences which is used as an momentous specie identification tool (Aliabad *et al.,* 2012; Bayatzadeh *et al.,* 2013; Ehtisham *et al.,* 2011; Lobova *et al.,* 2012; Jarquin *et al.,* 2009; Perez *et al.,* 2011). *M. synoviae* specific amplification revealed that out of 40 isolates of Mycoplasma, 20 (50%) was *M. synoviae*. Sequencing of specific PCR amplicons (213 bp) was used for confirmation and phylogenetic analysis. All *M. synoviae* isolates showed more than 99 % similarity to each other and other *M. synoviae*. Neighbor-Joining method is used to extrapolate evolutionary history (Saitou and Nei, 1987). Optimal tree with branch length sum of = 0.54344747 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to deduce the phylogenetic tree. The evolutionary distances were reckoned by the Maximum Composite Likelihood method (Tamura *et al*., 2004) and were in units of number of base substitutions per site. Variation rate among sites was modeled with a gamma distribution (shape parameter = 3). Phylogenetic analysis on 16S r DNA partial amplicons sequences includes a total of 5 sequences reported in this study and 07 outgroup. All positions with missing data and gaps were precluded. There were total of 197 positions in the final dataset.

M. synoviae partial amplification of vlhA gene is useful for *M*. synoviae strain identification (Dijkman *et al.,* 2014). Out of 20 isolates of *M. synoviae,* vlhA gene was successfully partially amplified from 05 (20%) isolates. These results are in agreement with ley, (2008) and Hammond *et al.,* (2009). For vlha partial amplification primers used by Wetzel *et al.,* (2010) were used. Phylogenetic tree, based on vlhA gene partial sequences revealed the evolutionary history of the isolates reported in this study. The Maximum Composite Likelihood method is used to reckon the evolutionary distances (Tamura *et al.,* 2004), and the number of base substitutions per site is used as unit. The analysis involved 16 nucleotide sequences with Codon positions 1st+2nd+3rd+Noncoding. All positions with missing data and gaps were closed out. There were total of 298 positions in the final data set. MEGA 6 is used to analyze evolution (Tamura *et al.,* 2013).

M. synoviae culture and its isolation is not an easy job but it is touchstone in the mycoplasma infection confirmation. Its phenotypical and genotypical characterization can help in future diagnostic and epidemiological studies.

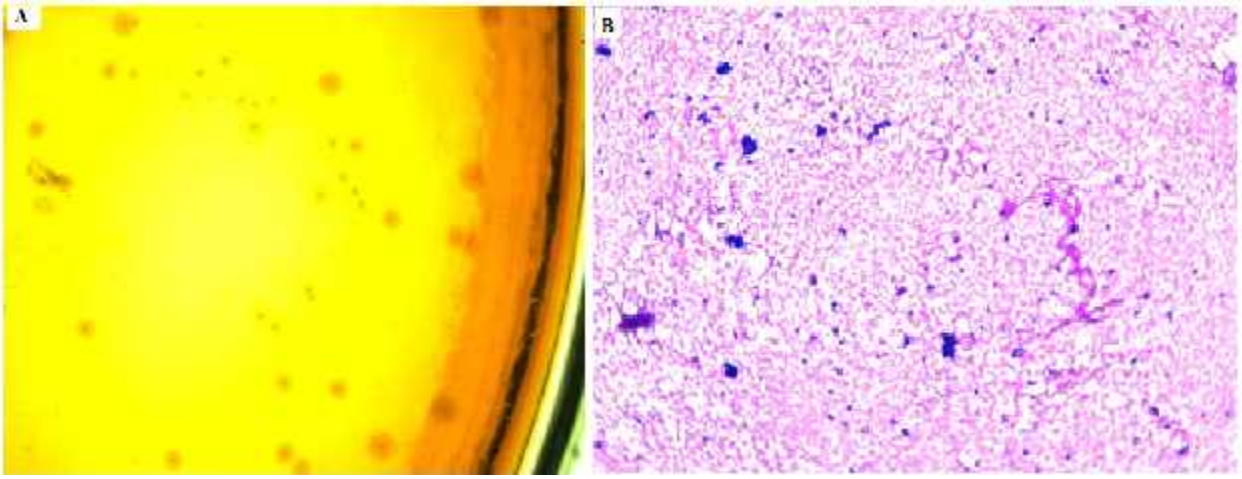

Figure 1: (A) Fried egg colonies of Mycoplasma, (B) Giemsa stained colonies of Mycoplasma

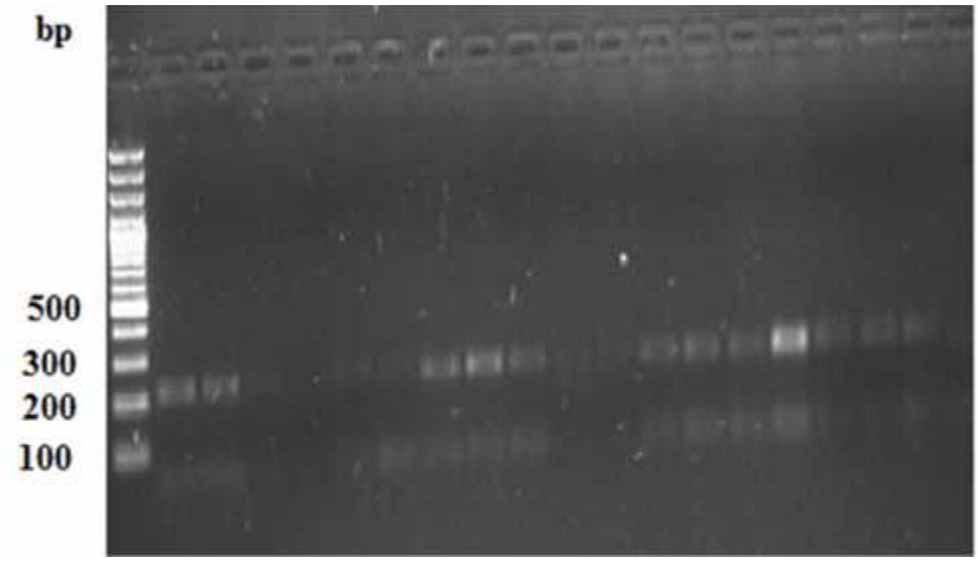

Figure 2: Partially amplified 16S ribosomal RNA gene of *M. synoviae*

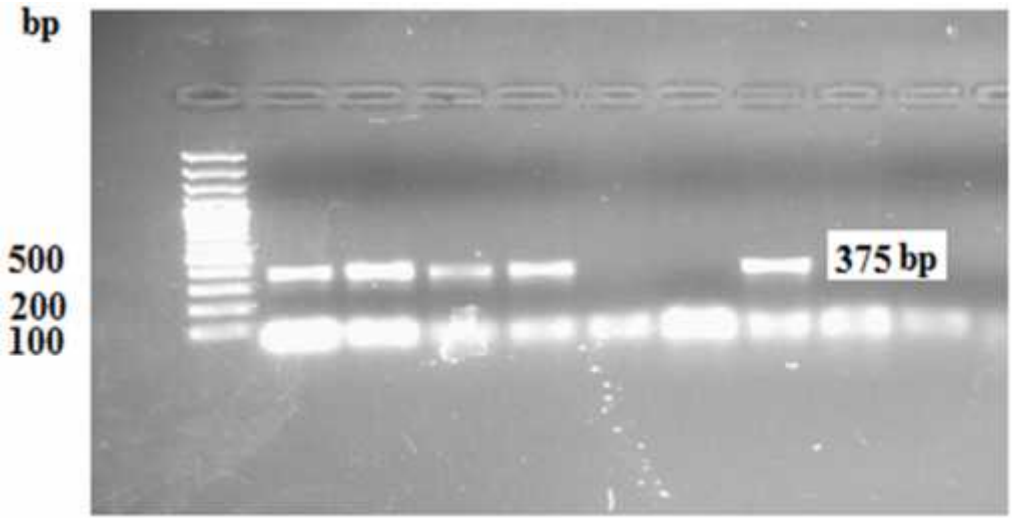

Figure 3: Partially amplified vlhA gene of *M. synoviae*

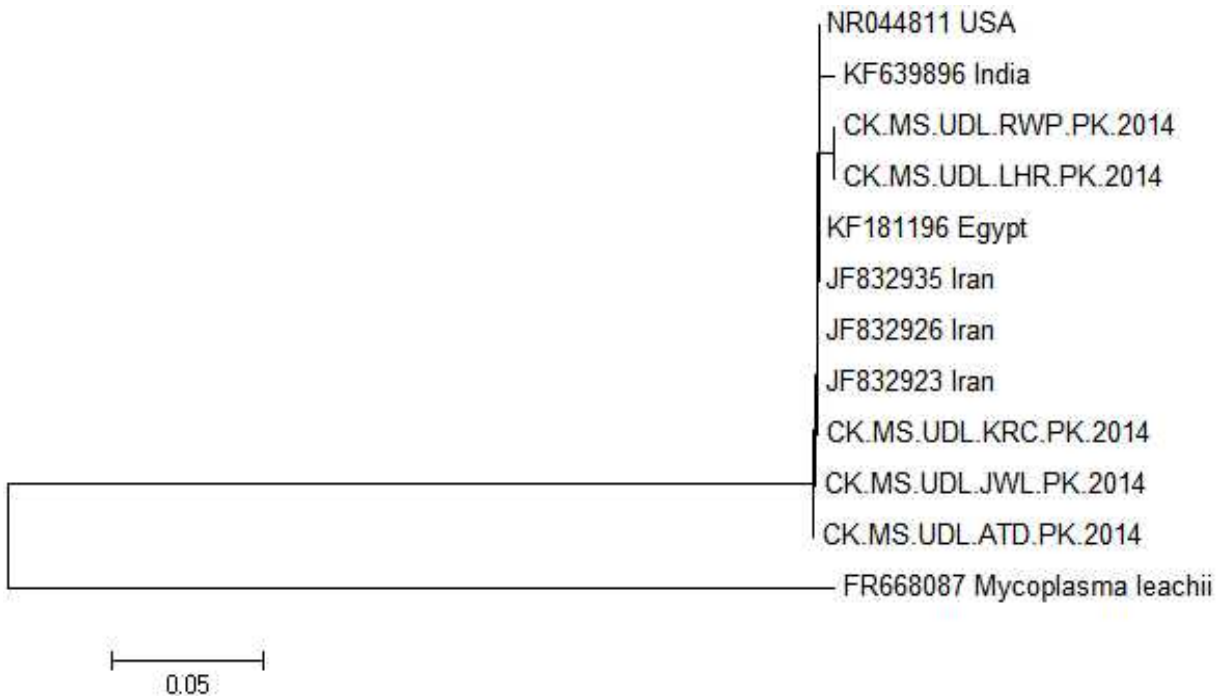

Figure 4. Phylogenetic tree of *M. synoviae* isolates based on 16S ribosomal RNA gene, partial sequence.

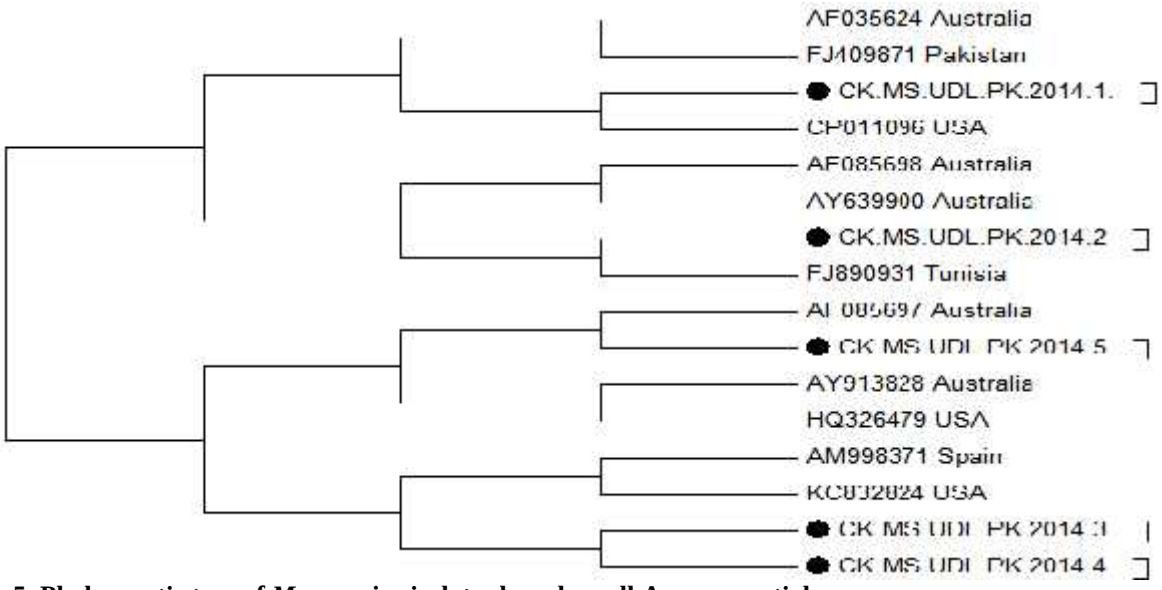

Figure 5. Phylogenetic tree of *M. synoviae* isolates based on vlhA gene, partial sequence

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