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Molecular characterization of Mycoplasma synoviae from backyard chickens in Dakahlia Governorate

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Abstract

M.S is considered the most common cause of respiratory and joint diseases in birds, which affects on poultry production specially in backyard sector which are not interested for vaccinations against such diseases. So one hundred blood samples were collected from diseased and apparently healthy birds from different localities in Dakahlia Governorate, Egypt to detect M.S antibodies by ELISA (indirect) test, results revealed that 23.0% of samples were positive. 17 swab samples from trachea and joint were cultured on specific media for M.S isolation but there were difficulty in culturing. M.S infection was confirmed by PCR which showed the presence of specific M.S specific gene. Sequencing and phylogentic analysis were applied for specific gene, phylogentic analysis of DNA sequence of M.S detected (M.synoviae-SB-Yard DG.019) revealed high homology with M.S strains M.synoviae-Eis 2-16 with a percentage of identity 99.05%. Heamatological and blood parameters of diseased bird with the detected M.S strains revealed heterophilia, lymphopenia, monocytosis eosinophilia basopenia increased total proteins, Creatinine, Uric acid, AST and ALT than un diseased birds.

Keywords: *Mycoplasma-synoviae, ELISA, PCR, Sequencing, phylogenetic tree, Leukocytes, total protein*

Introduction

Mycoplasma synoviae is a major pathogen of poultry throughout the world, causing chronic respiratory disease and sinusitis in infected chickens and turkeys **(Kleven, 1997),** it may result in an infection ranging from subclinical to sever (**Bencina *et al.,* 2001**) Arthritis and tenosynovitis of the hock and stifle joints are the most common skeletal affections in breeder chicken, in addition to spondylitis and osteomyelitis lesions (**Adair *et al.,* 1990**). MS is one of the most important bacterial pathogens causing arthritis. It is a worldwide economically important pathogen of poultry, causing respiratory affections, synovitis, abnormalities in eggshell, and drop in production in chickens and turkeys (**Catania *et al.,* 2010** & **Ferguson and Noormohammadi**, **2013** & **Jeon *et al.,* 2014** & **Sumitha**, **and Sukumar**, **2017**). Birds suffering from infectious synovitis are depressed, reluctant to walk with lameness, and affected hock joints are swollen and hot. The synovial membranes of tendon sheaths become thickened, edematous, with fibrinous exudates accumulation within and around the tendon sheaths (**Kieronczyk *et al.,* 2017**) MS is transmitted laterally and vertically, with lateral transmission through direct contact with infected carrier birds and fomites (**Stanley *et al.,* 2001**) MS infection in poultry flocks can be diagnosed either serologically, mainly done by serum plate agglutination (SPA) and hemagglutination inhibition, or through direct microbial isolation and identification that represent the gold standard, but time-consuming due to long incubation and fastidious nature of mycoplasmas (**Ferguson and Noormohammadi**, **2013**) Recently, molecular diagnosis with a polymerase chain reaction (PCR) is commonly used (**Uddin *et al.,* 2016**).

Literature on the epidemiology of avian mycoplasmosis in backyard chickens in Africa is

scanty, with few reports in Zimbaboye (**Kelly *et al.,* 1994**), Botswana (**Mushi *et al.,* 1999**), Benin (**Chrysostome *et al.,* 1995),** South Africa (**Thekisoe *et al.,* 2003**), and Ethiopia (**Chaka *et al.,* 2012**). Moracoo (**Saadia *et al.,* 2014**). The success of control programmers depends on accurate and timely diagnosis of infected flocks. Therefore, a diagnostic assay with high sensitivity, high specificity, and a fast detection time is required for monitoring M. synoviae in poultry flocks, (**Buntz *et al* 1986**, **Cookson *et al* 1994**), rapid diagnosis is needed to prevent dissemination of infection and has traditionally been achieved by serological screening for antibodies or by culture of the organism. Traditional culturing is the least favored because the it is time consuming, slow growing, and fastidious nature of MS strains. Serology is much faster than culturing, but non -specific and cross-reactions between species, miss-interpretations due to recent vaccination, and cost are all disadvantages (**ElShafey *et al.,* 2016**). MS strains can vary in antigenic make up and have the ability to alter the expression of major surface antigenic proteins affects the sensitivity and specificity of serologic monitoring systems (**Adair *et al* 1990**, **Avakian *et al* 1990**, **Bradly *et al* 1988**, **Noormohammadi *et al* 1997**). For these reasons, use of rapid and sensitive detection methods, like PCR method, can be advantageous (**Fernandez *et al* 1993**, **Hyman *et al* 1989**, **Kempf *et al* 1993**).

Sequencing of specific M.S–strains WVU1853 genome was the first version by **Meghan *et al*, (2015)** who made hybrid sequence assembly of this M.S type strain and compared to all strains of M.S ,their finding supported prior conclusions about M.S based on genome of that un characterized field strain and provide the evidence of epigenetic modification in M.S. The emergence of M. synoviae infectious synovitis in both chickens and turkeys associated with economic losses prompted the need for efficacious antibiotic treatments. In order to provide the optimum choice for antibiotic therapy, knowledge of the susceptibility profiles of available compounds was needed as underlined by (**Elshafey *et al.,* 2016**). (**Amer *et al.,* 2019**) mentioned that, four field isolates of MS were identified in examined broiler breeder flock and phylogenetic study of these isolates revealed the variation between isolated MS strains and vaccine strain and stated the requirement for evaluating the vaccine efficacy against the present field isolates of MS. Differential leukocytic count of birds after exposure to Mycoplasma species have been described also various blood parameters in commercial hens acutely and chronically infected with M.synoviae were determined by (**Branton *et-al.,* 1997).** Application of MS immunization of breeder flocks is necessary for proper control of the disease. **So the objective**, **of this work** is to study the seroprevalence of MS in some backyards chicken suffering from arthiritis and tenosynovitits in Egypt compare percentage with other in relation African countries then try to isolate M.S from clinical samples and the molecular diagnosis, sequencing and phylogenetic analysis of M.S specific gene, effect of these M.S specific strain on hematological parameter and total protein to study its effect on immunity and metabolism of diseased birds.

Materials and Methods

Case history and Post–mortem examination:

Five different backyard chicken rearing houses were randomly selected in different areas of Dakahlia Governorate, Live birds were observed for clinical case history of respiratory signs, Keel bursitis, swelling of hock, shank with bilateral asymmetrical swelling and un even size of birds. Post-mortem examination showed air saculities joints has grey to yellow exudate and swollen spleen with green liver.

Sampling: A total of 100 blood samples were collected from apparent healthy chickens in backyard groups not previously vaccinated to *Mycoplasma*. Approximately 5mL of blood was collected from wing vein as described by **Kelly and Alworth** (**2013**) for ELISA. In addition, 17 joint, tracheal swabs were collected from clinically diseased chickens showing clinical signs of suspected *Mycoplasma* infection, for isolation, molecular diagnosis of Mycoplasma synoviae and Sequencing, phylogenetic analysis of of specific pathogenic gene. Blood samples (with and without anticoagulant) for hematological and blood parameter investigation were also collected from clinically diseased chicken showing clinical signs.

Detection of M.S antibodies by Enzyme-linked immunosorbent assay (ELISA) as described by **Feberwee *et al.,* (2005)** using ELISA kits (Svanovir® MS-Ab kit, Boehringer Ingelheim Svanova, Uppsala, Sweden) according to the manufacturer's instructions. Results showed in Table (3).

B-Isolation and identification of MS:-

The collected tracheal and joint swab samples are propagated in liquid and solid media specific for isolation of Mycoplasma as described by (**Frey *et al.,* 1968**) and genus *determination and biochemical characterization* were carried out according to (**Erno** and **Stipkovits**, **1973**).

C - Molecular diagnosis of M.S (OIE,2008) by:-

Detection of 16SrRNA gene by specific primers (LAUERMAN *et al.*, (1998).

Primers used for MS detection and Cycling procedures as showed in table (1)

Table (1). Primers and cycling procedures for detection of *Mycoplasma synoviae16S rRNA*

Target gene	Primers sequences 5′–3′	Amplified segment (bp)	Primary Denaturation	Amplification (40 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
16Sr RNA	F.5'-GAG-AAG-CAA-AAT-AGT-GAT-ATC-A-3'	211 bp	94˚C5 min.	94˚C 30 sec.	55˚C 30sec.	72˚C 60sec.	72˚C 5min.	**Lauernan, L.H. (1998).**
	R: 5'-CAG-TCG-TCT-CCG-AAG-TTA-ACA-A-3'							

-Amplification with specific primers: (Zhao, S. and R. Yamamoto, 1993a):

In this study two published primer sets were used for the specific detection of genus and species of *M. synoviae* as showed in table (2)

Table (2). Primers and cycling procedures for detection of *Mycoplasma synoviae* specific gene

Target gene	Primers sequences 5′–3′	Amplified segment (bp)	Primary denaturation	Amplification (35cycles)			Reference
				Secondary denaturation	Annealing	Extension	
	′5-GAA GGT ATT TAA TCA ACC TTG GCTG3	1000bp	95˚C 2min.	94˚C 20 sec.	51˚C 30sec.	72˚C 1min.	**Zhao, S. and R. Yamamoto, (1993)**
	5′TGT AAT GGC TCC AGC TCA AGC AGC T3′).						

D- Mycoplasma Synoviae sequencing and phylogenetic analysis:

The amplified fragments were purified using Gene Jet PCR purification kit; Fermentas (Cat no. KO701) as following:-

1- Add a 45ul of Binding Buffer to completed PCR mixture. Mix thoroughly.

2- Transfer the mixture from step 1 to the Gene JET™ purification column. Centrifuge for 30-60 s at > 12000 x g.

Discard the flow-through.

3- Add 100ul of Wash Buffer to the Gene JET™ purification column. Centrifuge for 30-60 s. Discard the flow-through and place the purification column back into the collection tube.

4- Centrifuge the empty Gene JET™ purification column for an additional 1 min to completely remove any residual wash buffer.

5- Transfer the Gene JET™ purification column to a clean 1.5 ml micro centrifuge tube. Add 25ulof Elution Buffer to the center of the Gene JET™ purification column membrane and centrifuge for 1 min.

6- Discard the Gene JET™ purification column and store the purified DNA at -20°C.

Published *strains in* GenBank were selected as Reference sequences. Sequencing was performed by sequencing to the PCR product on GATC Company by use ABI 3730xl DNA sequencer by using forward and reverse primers. Only by combining the traditional Sanger technology with the new 454 technology, can genomes now be sequenced and analyzed in half the usual project time, with a considerable reduction in the number of coatings and gaps. In addition, considerable cost advantages now make genome sequencing with the 454 technology accessible to the research community.
and identification of homologies between nucleotide and amino acid sequences our strains were compared with other strains published on GenBank using BLAST 2.0 and PSI-BLAST search programs, (National Center for Biotechnology Information NCBI"http:// www.ncbi.nlm.nih.gov/), respectively The obtained nucleotide sequences comparisons and their multiple alignments with reference *strains* as well as the deduction of amino acidequences were done using the BioEdit sequence alignment editor (**Hall and Bio**,**1999**), CLUSTALX software for multiple sequence alignment [Nicholas and Ayling, 2003], ClustalW software for multiple sequence alignment (**Thompson *et al*., 1994**), ClustalV (**Higgins and sharp**, **1989**) and MegAlign (DNASTAR, Lasergene®, Version 7.1.0, USA) (**Kumar *et al*., 2004**).The phylogenetic trees were constructed using MegAlign for tree reconstruction of sequences by Neighbor-joining method based on ClustalW. Bootstrapping values were calculated using a random seeding value of 111 (**Thompson *et al*., 1994**). ClustalV was used when end gaps were faced. Sequence divergence and identity percent were calculated by MegAlign The structural character of our protein sequence was identified by Protean (DNASTAR, Lasergene®,Version 7.1.0. USA)

E- Hematological investigation of clinically diseased birds with M.S:

-The blood samples with EDTA were analyzed for total and differential leukocytic count (DLC) using the methods described by **Jain (1986)**.

-Determination of liver enzymes (AST, ALT) and kidney function test (creatinine, uric acid), Albumin, and total protein**:** All biochemical tests were measured calorimetrically using a commercial kits of BioMed-Diagnostic, on a semi-automated Photometer (5010 V5+, RIE-LE GmbH & Co, Berlin, Germany). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured according to the method of **Reitman and Frankel (1957)**, serum albumin level was estimated by the method of **Doumas *et al.* (1981)**. Serum uric acid was measured by the method previously obtained by **Sanders** and **Pasman (1980)**. Serum total proteins and creatinine levels were determined, serum albumin level was estimated by the method of **Doumas *et al.* (1981)**.

Results

A-Detection of Mycoplasma synoviae antibodies: Sero-prevalence by ELISA test for detecting *Mycoplasma synoviae* antibodies showed that from 100 serum samples examined 23% (23/100) of samples were positive.

Table (3). Results of *Mycoplasma synoviae* antibodies by ELISA:-

Backyard	Age	Clinical signs	Number of samples	ELISA Positive	%
5	6 - 16 weeks	respiratory, locomotor disorders , growth troubles	100	23	23.0

B- Isolation and identification of Mycoplasma synoviae :

-regarding traditional method for isolation from tracheal and synovial swabs, isolation were difficult and the results showed that *Mycoplasma synoviae* was confirmed using PCR targeting 16s rRNA gene.

-Preparation of clinically diseased tracheal and joint samples (photo. 1, 2) collected showed that, five (5/17) samples were positive for preparation of suspected Mycoplasma positive samples in broth with a percentage of (29.4%).

Photo (1, 2): Leg chicken with swollen joint containing caseous exudates from where *Mycoplasm* synoviae (MS) diagnosed.

C- Molecular diagnosis of M.S :-

-results of 16SrRNA amplification for five field samples for M.S and Gel Electrophoresis Amplified DNA was separated by electrophoresis in 1.5% agarose (Pharmacia ,Biotech) AB, Uppsala, Sweden) gels, post stained with ethidium bromide, illuminated with ultraviolet light, and photographed. Figure (1) which revealed three positive MS field samples out of five extracted DNA sample

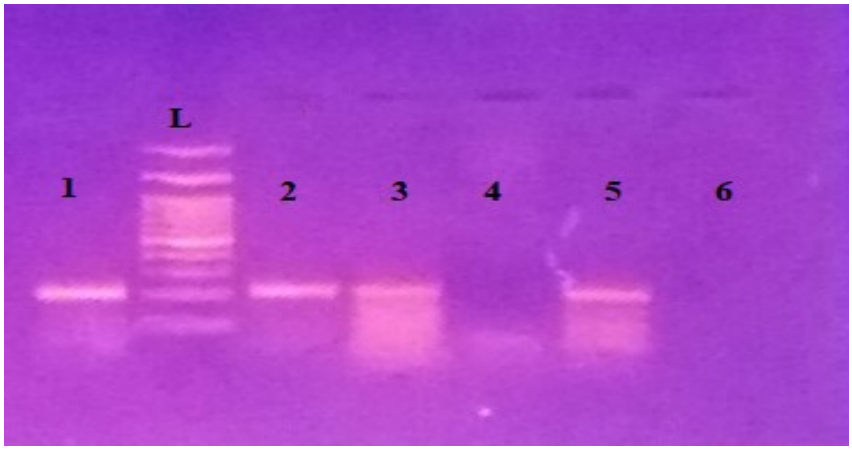

Figure (1): Agarose gel electrophoresis pattern for amplification product of 16s rRNA gene of phenotypically identified *Mycoplasma synoviae* isolates; 2, 3, 5; positive isolates, 4; negative isolate, 6; Negative control, 1; Positive control, L: Ladder; DNA molecular size marker (100bp)

Results of amplification for specific MS gene at 1000bp:- Result in figure (2) showed two positive MS field sample by PCR using specific gene.

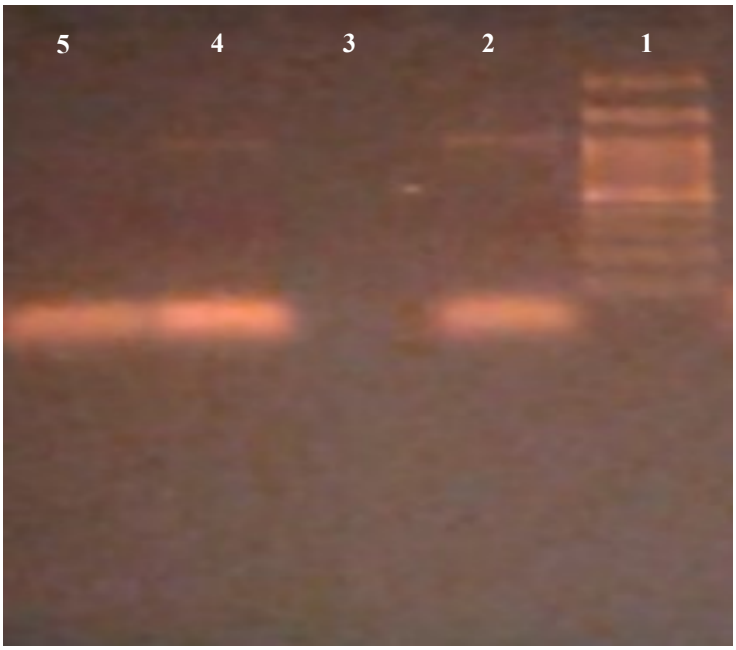

Figure (2): Electrophoretic agarose gel of MS specific gene
1- 100bp ladder
2- MS positive control
3- negative control
4- positive sample 1000 bp
5- positive sample 1000 bp

-Nucleotide sequence alignment of specific gene of MS field isolate

CLUSTAL O(1.2.4) multiple sequence alignment.

https://drive.wps.com/d/AKxg9pi64YwngICA3bDGEwClustal .o

Phylogenetic analysis of DNA sequence of M.S detected (M.synoviae-SB-YardDG.019) revealed high homology with the M.S strains M.synoviae-Eis2-16 with a percentage of identity 99.05% and with a percentage of identity 99.47% with M.synoviae-ATCC-5204. (Figure 3 & 4).

D- Mycoplasma synoviae Sequencing and phylogenetic analysis

- Nucleotide sequence alignment of specific gene of MS field isolate

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CLUSTAL O(1.2.4) multiple sequence alignment.

M.synoviae-ATCC-25204 tctatcgtttgtaattgcagaaactgcaaaatacactgcttcag-tatcacctttattt 59
M.synoviae-NCTC10124  tctatcgtttgtaattgcagaaactgcaaaatacactgcttcag-tatcacctttattt 59
M.synoviae-WVU1853  ------------------------------------------------------tttattt  7
M.synoviae-SB-YardDG.019--ttcagattcatct--ggatcatcagaacaaacaccaccaagtacaacttaataata 54
M.synoviae-Eis2-16  ---ttcagattcatct---ggatctacagaacaaacaccaccaagtacaacttaataatc  54
M.synoviae-ATCC-5204 ---ttcagattcatct---ggatctacagaacaaacaccaccaagtacaacttaataata 54
                           ** *
M.synoviae-ATCC-25204 gttgcaagaatacgatgtccttattgctt-agtaaatc--gtctaaagttcctggtctaa 116
M.synoviae-NCTC10124 gttgcaagaatacgatgtccttattgctt-agtaaatc--gtctaaagttcctggtctaa  116
M.synoviae-WVU1853   gttgcaagaatacgatgtccttattgctt-agtaaatc--gtctaaagttcctggtctaa  64
M.synoviaeSB-YardDG019ataaaaccaaaaacatgccccaaaatgttatccctatactgagacgggtttatgccaaaa 114
M.synoviae-Eis2-16 ataaaaccaaaaacatgccccaaaatgttatccctatactgagacgggtttatgccaaaa   114
M.synoviae-ATCC-5204ataaaaccaaaaacatgccccaaaatgttatccctatactgagacgggtttatgccaaaa  114
                 *   *  ** *  *** **  *    **      **   *      ***  **    **
M.synoviae-ATCC-2520 gttcttctctttttgttttttatttttttcaac---ttta-gttatagtaactccgttt  172
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M.synoviae-NCTC10124 gttcttctctttttttgttttttatttttttcaac---ttta-gttatagtaactccgttt 172
M.synoviae-WVU1853  gttcttctctttttttgttttttatttttttcaac---ttta-gttatagtaactccgttt  120
M.synoviae-SBYardDG.019 attactcttgaaagttttactgcatcgcaaacaaatgtagaagataataaatcaaaac 172
M.synoviae-Eis2-1 attactcttgaaagttttactgcatcgcaaacaaatgtagaagataataaatcaaaac      172
M.synoviae-ATCC-5204  a-ttactcttgaaagttttactgcatcgcaaacaaatgtagaagataataaatcaaaac 172
             * *****    *****      *    ***   * **    *** *** **

M.synoviae-ATCC-25204 tttgaatttgttacaaattcagctttaaatttagggtcaaatttattaacatagttaatt 232
M.synoviae-NCTC10124  tttgaatttgttacaaattcagctttaaatttagggtcaaatttattaacatagttaatt 232
M.synoviae-WVU1853   tttgaatttgttacaaattcagctttaaatttagggtcaaatttattaacatagttaatt 180
M.synoviae-SB-YardDG.019  ttcaaaaatg--------atttaatactaaagcaaattgaagcaatctagaagatc 220
M.synoviae-Eis2-16     ttcaaaaatg----------atttaatactaaagcaaattgaagcaatctagaagatc 220
M.synoviae-ATCC-5204  ttcaaaaatg-----------atttaatactaaagcaaattgaagcaatctagaagatc 220
             **  **  ** *****   **  *  ** *  *  **  ***   **

M.synoviae-ATCC-25204 ac----------agacttc--aataaaggatttggatatctattggtaaatagcagtccac 281
M.synoviae-NCTC10124 ac----------agacttc--aataaaggatttggatatctattggtaaatagcagtccac  281
M.synoviae-WVU1853   ac----------agacttc--aataaaggatttggatatctattggtaaatagcagtccac  229
M.synoviae-SBYardDG019aacttgttaaaaaactaggaagtaagaaatttaaaaacgttaaactaaccaatccacaag 280
M.synoviae-Eis2-16  aacttgttaaaaaactaggaagtgagaaatttaaaaacgttaaactaaccaatccacaag  280
M.synoviae-ATCC-5204 aacttgttaaaaaactaggaagtaagaaatttaaaaacgttaaactaaccaatccacaag 280
                   * *** * * *   ****  * *  * ***  *  * *

M.synoviae-ATCC-25204 tagttggatttcctctaaatgcattgttaaat---gcacctgtaacatttgatgttccat 338
M.synoviae-NCTC10124  tagttggatttcctctaaatgcattgttaaat---gcacctgtaacatttgatgttccat 338
M.synoviae-WVU1853  tagttggatttcctctaaatgcattgttaaat---gcacctgtaacatttgatgttccat  286
M.synoviae-SBYardDG019tagattgagacatagtaaatagcaaccaatatagacctaaagttacctttgatgtaaccc 340
M.synoviae-Eis2-16   tagattgagacatagtaaatagcaaccaatatagacctaaagttacctttgatgtaaccc  340
M.synoviae-ATCC-5204  tagattgagacatagtaaatagcaaccaatatagacctaaagttacctttgatgtaaccc 340
             ***   *****   * **    *    ** ** ********  *

M.synoviae-ATCC-25204taattgaagtattttcttgtccgcctactgttggcagatctgcatctaagacaatatttg  398
M.synoviae-NCTC10124 taattgaagtattttcttgtccgcctactgttggcagatctgcatctaagacaatatttg  398
M.synoviae-WVU1853  taattgaagtattttcttgtccgcctactgttggcagatctgcatctaagacaatatttg   346
M.synoviaeSBYardDG.019aaaaagaaggttatgaattgcaaagaggaagtgaattttcacaaagtcttacaatggtta 400
M.synoviae-Eis2-16   aaaaagaaggttatgaattgcaaagcggaagtgaattttcacaaagtcttacaatggtta  400
M.synoviae-ATCC-5204 aaaaagaaggttatgaattgcaaagcggaagtgaattttcacaaagtcttacaatggtta  400
           **  ****  * *   *  *          **     **   *  *   *****  **

M.synoviae-ATCC-25204  gcccagtatagttaagatatacattaa--------cagttcttaaaacatttgcatcgc 449
M.synoviae-NCTC10124  gcccagtatagttaagatatacattaa----------cagttcttaaaacatttgcatcgc 449
M.synoviae-WVU1853    gcccagtatagttaagatatacattaa----------cagttcttaaaacatttgcatcgc 397
M.synoviaeSB-YardDG019taagagttttatatacaactcaatcacctaatgcaaacgttttacaaatgcaaggagctt 460
M.synoviae-Eis2-16 taagagttttatatacaactcaatcacctaatgcaaacgttttacaaatgcaaggagctt    460
M.synoviae-ATCC-5204 taagagttttatatacaactcaatcacctaatgcaaacgttttacaaatgcaaggagctt 460
              *** *  *  * *  *  **  *** *  ***     * * *

M.synoviae-ATCC-25204 taactgttgtagtgtcattaggcactgctaaagatgaagctccttgcatttgtaaaacgt 509
M.synoviae-NCTC10124  taactgttgtagtgtcattaggcactgctaaagatgaagctccttgcatttgtaaaacgt 509
M.synoviae-WVU1853   taactgttgtagtgtcattaggcactgctaaagatgaagctccttgcatttgtaaaacgt  457
M.synoviaeSBYardDG.019catctttagcagtgcctaatgacactacaacagttagcgatgcaaatgttttaagaactg 520
M.synoviae-Eis2-16  catctttagcagtgcctaatgacactacaacagttagcgatgcaaatgttttaagaactg  520
M.synoviae-ATCC-5204 catctttagcagtgcctaatgacactacaacagttagcgatgcaaatgttttaagaactg 520
                  * ** * * **** *    * **** * * ** *   * * *     ***  * ***

M.synoviae-ATCC-25204 ttgcattaggtgattgagttgtatataaaactcttataaccattgtaagactttgtgaaa 569
M.synoviae-NCTC10124  ttgcattaggtgattgagttgtatataaaactcttataaccattgtaagactttgtgaaa 569
M.synoviae-WVU1853    ttgcattaggtgattgagttgtatataaaactcttataaccattgtaagactttgtgaaa 517
M.synoviae-SB-YardDG.019ttaatgtat--------atcttaactatactgggccaaatattgtcttag--atgcag 568
M.synoviae-Eis2-16    ttaatgtat---------atcttaactatactgggccaaatattgtcttag---atgcag 568
M.synoviae-ATCC-5204  ttaatgtat---------atcttaactatactgggccaaatattgtcttag---atgcag 568
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M.synoviae-ATCC-25204 attcacttccgctttgcaa---ttcataaccttcttttttgggttacatcaaaggtaactt 626
M.synoviae-NCTC10124  attcacttcctctttgcaa---ttcataaccttcttttttgggttacatcaaaggtaactt 626
M.synoviae-WVU1853  attcacttcctctttgcaa---ttcataaccttcttttttgggttacatcaaaggtaactt  574
M.synoviaeSBYardDG.019atctgccaacagtaggcggacaagaaaatacttcaattaatggaacatcaaatgttaca- 627
M.synoviae-Eis2-16  atctgccaacagtaggcggacaagaaaatacttcaattaacggaacttcaaatgtaaca-  627
M.synoviae-ATCC-5204  atctgccaacagtaggcggacaagaaaatacttcaattaatggaacatcaaatgttaca- 627
            **   *   *  *  **        * *  ****  **   *  ** ***** ** **

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M.synoviae-NCTC10124 taggtctatattggttgctatttactatgtctcaatctacttgtggattggttagtttaa  686
M.synoviae-WVU1853   taggtctatattggttgctatttactatgtctcaatctacttgtggattggttagtttaa  634
M.synoviae-SB-YardDG.019ggtgcatttaacaatgcatttagaggaaatccaactagtggactgctatttaccaata 685
M.synoviae-Eis2-16    ggtgcatttaacaatgcatttagaggaaatccaactagtggactgctatttaccaata  685
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M.synoviae-ATCC-5204 ggtgcatttaacaatgcatttagaggaaatccaactagtggactgctatttaccaata    685
          ***  ** *        *****       ** * *** * *     *  ***     *

M.synoviae-ATCC-25204 cgtttttaaatttcttacttcctagtttttttaacaagttgatcttctagattgcttcaat 746
M.synoviae-NCTC10124  cgtttttaaatttcttacttcctagtttttttaacaagttgatcttctagattgcttcaat 746
M.synoviae-WVU1853   cgtttttaaatttcttacttcctagtttttttaacaagttgatcttctagattgcttcaat  694
M.synoviae-SB-YardDG.019 gatatccaaatccttta--------ttgaagtctgtaattaactatgttaataaatt 734
M.synoviae-Eis2-16  gatatccaaatccttta-----------ttgaagtctgtaattaactatgttaataaatt   734
M.synoviae-ATCC-5204  gatatccaaatccttta-----------ttgaagtctgtaattaactatgttaataaatt 734
              * *  ****   ***           ** **     * **   ***  **  *  * *

M.synoviae-ATCC-25204 ttgctttagtattaa------------atcatttttgaagttttgatttattatcttcta 794
M.synoviae-NCTC10124 ttgctttagtattaa------------atcatttttgaagttttgatttattatcttcta  794
M.synoviae-WVU1853   ttgctttagtattaa------------atcatttttgaagttttgatttattatcttcta  742
M.synoviaeSBYardDG.019tgaccctaaatttaaagctgaatttgtaacaaattcaaaaaacggagttactataact-a 793
M.synoviae-Eis2-16 tgaccctaaatttaaagctgaatttgtaacaaattcaaaaaacggagttactataact-a    793
M.synoviae-ATCC-5204 tgaccctaaatttaaagctgaatttgtaacaaattcaaaaaacggagttactataact-a  793
             *  *  **   *** * **  **  **     ** *** ***     *
M.synoviae-ATCC-25204 catttgtttgcgatgcagtaaaactttcaagagtaatttttggcataaacccgtctcagt 854
M.synoviae-NCTC10124 catttgtttgcgatgcagtaaaactttcaagagtaatttttggcataaacccgtctcagt  854
M.synoviae-WVU1853  catttgtttgcgatgcagtaaaactttcaagagtaatttttggcataaacccgtctcagt   802
M.synoviae-SB-YardDG.019 aagttgaaaaaaatagaaaacaaaaaagagaagaacttagaccaggaactttagacg 850
M.synoviae-Eis2-16    aagttgaa---aaaaatagaaaaacaaaaaagagaagaacttagaccaggaactttagacg 850
M.synoviae-ATCC-5204  aagttgaa---aaaaatagaaaaacaaaaaagagaagaacttagaccaggaactttagacg 850
                           * ***      *      *****    ***** *    ** *   *    * *   *
M.synoviae-ATCC-25204 atagggataacattttggggcatgtttttggttttattattattaagttgtacttggtgg 914
M.synoviae-NCTC10124 atagggataacattttggggcatgtttttggttttattattattaagttgtacttggtgg  914
M.synoviae-WVU1853   atagggataacattttggggcatgtttttggttttattattattaagttgtacttggtgg  862
M.synoviaeSBYardDG.019attta-ctaagcaataaggacatcgtattcttgcaacaaataaaagg-tgatactgaagc 908
M.synoviae-Eis2-16 attta-ctaagcaataaggacatcgtattcttgcaacaaataaaagg-tgatactgaagc    908
M.synoviae-ATCC-5204 attta-ctaagcaataaggacatcgtattcttgcaacaaataaaagg-tgatactgaagc  908
             **     ***    *  ** ***  * **  *   *  * **  * * **    **  *
M.synoviae-ATCC-25204  tgtttgttctgtagatccagatg--aatc--tgaattactatatccagctggttgtaat 969
M.synoviae-NCTC10124  tgtttgttctgtagatccagatg---aatc--tgaa------------------------ 945
M.synoviae-WVU1853   tgtttgttctgtagatccagatg---aatc--tgaa------------------------  893
M.synoviaeSBYardDG.019agtgtattttgcagtttctgcaattacaaacgatagatgacttacaccgttacttgtaag 968
M.synoviae-Eis2-16   agtgtattttgcagtttctgcaattacaaacgataga----------------------- 945
M.synoviae-ATCC-5204  agtgtattttgcagtttctgcaattacaaacgatagatgacttaacaccgtacttgtaag 968
                           ** * ** ** ** * * *       *      *
```

https://drive.wps.com/d/AKxg9pi64YwngICA3bDGEwClustal .o

-Phylogenetic tree of MS isolates based on the nucleotide sequence of specific gene:-

Figure (3) . Phylogenetic tree of MS isolates based on the nucleotide sequence of specific gene.

Percent Identity Matrix - created by Clustal2.1

1: M.synoviae-ATCC-25204	100.00	99.89	99.89	45.81	45.75	45.8
2: M.synoviae-NCTC10124	99.89	100.00	100.00	45.80	45.80	45.91
3: M.synoviae-WVU1853	99.89	100.00	100.00	45.57	45.45	45.57
4: M.synoviae-SB-YardDG.019	45.81	45.80	45.57	100.00	99.05	99.07
5: M.synoviae-Eis2-16	45.75	45.80	45.45	99.05	100.00	99.47
6: M.synoviae-ATCC-5204	45.81	45.91	45.57	99.07	99.47	100.00

b- Amino acid sequences alignment of specific MS gene:

(CLUSTAL O(1.2.4) multiple sequence alignment:-

```
M.synoviae-SB-YardDG.019      FRFIWIIRTNTTKYNLIIIKPKTCPKMLSLYWDGFMPKITLESFTASQTNVEDNKSKLQK 60
M.synoviae-NCTC10124          FRFIWIYRTNTTKYNLIIIKPKTCPKMLSLYWDGFMPKITLESFTASQTNVEDNKSKLQK 60
M.synoviae-WVU1853            FRFIWIYRTNTTKYNLIIIKPKTCPKMLSLYWDGFMPKITLESFTASQTNVEDNKSKLQK 60
M.synoviae-ATCC-25204         FRFIWIYRTNTTKYNLIIIKPKTCPKMLSLYWDGFMPKITLESFTASQTNVEDNKSKLQK 60
M.synoviae-ATCC-5204          FRFIWIYRTNTTKYNLIIIKPKTCPKMLSLYWDGFMPKITLESFTASQTNVEDNKSKLQK 60
M.synoviae-Eis2-16            FRFIWIYRTNTTKYNLIIIKPKTCPKMLSLYWDGFMPKITLESFTASQTNVEDNKSKLQK 60
                              ****** **************************************************

M.synoviae-SB-
YardDG.019      WFNTKANWSNLEDQLVKKLGSKKFKNVKLTNPQVDWDIVNSNQYRPKVTFDVTQKEGYEL 120
M.synoviae-NCTC10124 WFNTKANWSNLEDQLVKKLGSKKFKNVKLTNPQVDWDIVNSNQYRPKVTFDVTQKEGYEL 120
M.synoviae-WVU1853    WFNTKANWSNLEDQLVKKLGSKKFKNVKLTNPQVDWDIVNSNQYRPKVTFDVTQKEGYEL 120
M.synoviae-ATCC-25204 WFNTKANWSNLEDQLVKKLGSKKFKNVKLTNPQVDWDIVNSNQYRPKVTFDVTQKEGYEL 120
M.synoviae-ATCC-5204  WFNTKANWSNLEDQLVKKLGSKKFKNVKLTNPQVDWDIVNSNQYRPKVTFDVTQKEGYEL 120
M.synoviae-Eis2-16    WFNTKANWSNLEDQLVKKLGSEKFKNVKLTNPQVDWDIVNSNQYRPKVTFDVTQKEGYEL 120
                      **********************:***************************************

M.synoviae-SB-YardDG.019 QRGSEFSQSLTMVIRVLYTTQSPNANVLQMQGASSLAVPNDTTTVSDANVLRTVNVYLNY 180
M.synoviae-NCTC10124     QRGSEFSQSLTMVIRVLYTTQSPNANVLQMQGASSLAVPNDTTTVSDANVLRTVNVYLNY 180
M.synoviae-WVU1853       QRGSEFSQSLTMVIRVLYTTQSPNANVLQMQGASSLAVPNDTTTVSDANVLRTVNVYLNY 180
M.synoviae-ATCC-25204 QSGSEFSQSLTMVIRVLYTTQSPNANVLQMQGASSLAVPNDTTTVSDANVLRTVNVYLNY 180
M.synoviae-ATCC-5204     QSGSEFSQSLTMVIRVLYTTQSPNANVLQMQGASSLAVPNDTTTVSDANVLRTVNVYLNY 180
M.synoviae-Eis2-16       QSGSEFSQSLTMVIRVLYTTQSPNANVLQMQGASSLAVPNDTTTVSDANVLRTVNVYLNY 180
                         ************************************************************

M.synoviae-SB-YardDG.019    TGPNIVLDADLPTVGGQENTSINGTSNVTGAFNNAFRGNPTSGLLFTNRYPNPLLKSVIN 240
M.synoviae-NCTC10124        TGPNIVLDADLPTVGGQENTSINGTSNVTGAFNNAFRGNPTSGLLFTNRYPNPLLKSVIN 240
M.synoviae-WVU1853          TGPNIVLDADLPTVGGQENTSINGTSNVTGAFNNAFRGNPTSGLLFTNRYPNPLLKSVIN 240
M.synoviae-ATCC-25204       TGPNIVLDADLPTVGGQENTSINGTSNVTGAFNNAFRGNPTSGLLFTNRYPNPLLKSVIN 240
M.synoviae-ATCC-5204        TGPNIVLDADLPTVGGQENTSINGTSNVTGAFNNAFRGNPTSGLLFTNRYPNPLLKSVIN 240
M.synoviae-Eis2-16          TGPNIVLDADLPTVGGQENTSINGTSNVTGAFNNAFRGNPTSGLLFTNRYPNPLLKSVIN 240
                            ************************************************************

M.synoviae-SB-YardDG.019  YVNKFDPKFKAEFVTNSKNGVTITKVEKNRKQKREELRPGTLDDLLSNKDIVFLQQIKGD 300
M.synoviae-NCTC10124      YVNKFDPKFKAEFVTNSKNGVTITKVEKNKKQKREELRPGTLDDLLSNKDIVFLQQIKGD300
M.synoviae-WVU1853        YVNKFDPKFKAEFVTNSKNGVTITKVEKNKKQKREELRPGTLDDLLSNKDIVFLQQI---  297
M.synoviae-ATCC-25204     YVNKFDPKFKAEFVTNSKNGVTITKVEKNKKQKREELRPGTLDDLLSNKDIVFLQQIKGD 300
M.synoviae-ATCC-5204      YVNKFDPKFKAEFVTNSKNGVTITKVEKNKKQKREELRPGTLDDLLSNKDIVFLQQIKGD 300
M.synoviae-Eis2-16        YVNKFDPKFKAEFVTNSKNGVTITKVEKNKKQKREELRPGTLDDLLSNKDIVFLQQIKGD  300
                          ***************************:********************************
```

Phylogenetic tree based on Amino acid sequences:-

Figure (4): Phylogenetic tree based on Amino acid sequences

Percent identity Matrix - created by Clustal2.1

1: M.synoviae-SB-YardDG.019	100.00	99.37	99.33	99.05	98.14	98.73
2: M.synoviae-NCTC10124	99.37	100.00	100.00	99.68	99.68	99.37
3: M.synoviae-WVU1853	99.33	100.00	100.00	99.66	99.66	99.33
4: M.synoviae-ATCC-25204	99.05	99.68	99.66	100.00	100.00	99.68
5: M.synoviae-ATCC-5204	98.14	99.68	99.66	100.00	100.00	99.68
6: M.synoviae-Eis2-16	98.73	99.37	99.33	99.68	99.68	100.00

**Phylogenetic analysis of DNA sequence of M.S detected (M.synoviae-SB-YardDG.019) revealed high homology with the M.S strains M.synoviae-Eis2-16 with a percentage of identity 99.05% and with a percentage of identity 99.47% with M.synoviae-ATCC-5204. (Figure 3 &4).

E– Results of hematological and blood parameters investigation:

Table (4). Results of hematological and blood parameters of clinically diagnosed birds positive for Mycoplasma synoviae.

Parameters	Blood from birds Positive for diagnosis of Mycoplasma synoviae	Blood from Negative birds for diagnosis of Mycoplasma synoviae
Total number of leukocytes (10^3/mm^3)	47.300 ± 4.000	31.000 ± 1.500
Eosinophil (10^3/mm^3)	826 ±50	1.145 ± 150
Basophil (10^3/mm^3)	40 ± 15	130 ± 50
Neutrophil (10^3/mm^3)	12.200 ± 100	6.250 ± 300
Lymphocyte (10^3/mm^3)	13.040 ± 269	29.000 ± 500
Monocyte (10^3/mm^3)	3.150 ± 200	1.500 ± 117
Albumin (g/dl) **T. proteins (g/dl)**	4.89± 0.50 7.34 ± 0.50	3.59± 0.25 5.99 ± 0.50
Uric acid (mg/dl)	6.43 ± 0.20	3.55 ± 0.50
Creatinine (mg/dl)	2.39 ± 0.30	1.04 ± 0.20
AST (IU/ml)	54.27±0.42	36.16 ± 1.69
ALT (IU/ml)	19.07 ± 0.09	11.99 ± 0.09

Discussion

A presumptive diagnosis of M. synoviae may be made on the basis of pale comb, droopiness, emaciation, leg weakness, breast blisters, enlarged foot pads or hock joints, swelling of hock, shank with bilateral asymmetrical swelling and un even size of birds. Post-mortem examination showed air saculities, joints has grey to yellow exudate and swollen spleen (splenomegaly) with enlarged liver and kidney. The backyard chickens tested in this study had no previous history of avian mycoplasmosis vaccination. Hence, the presence of antibodies to *Mycoplasma synoviae* (MS) in all surveyed backyards was considered clear evidence that the birds have been naturally exposed to this infectious agent as shown in photo(1, 2). **OIE, (2008**) recommends the use of serological tests for avian mycoplasmosis only as screening tools in the diagnosis of flocks, not of individual birds. The current study used ELISA test as serological test for detection specific antibody

against MS, prevalence of MS observed in this study was 23.0% as showed in table (3), the same results were recorded by **Majid *et al.*, (2015)** who detected MS antibodies with prevalence of 27.0%. Lowered results were recorded by (**Sun *et al.*, 2017)** who detected MS antibodies with a percentages of 16.9 %.

In addition, regarding isolation and identification of *Mycoplasma synoviae* from diseased chicken seventeen joint swab samples were used to try to isolate *Mycoplasma synoviae* on specific medium as described by **Frey *et al,*** (**1968**), isolation were difficult A PCR assay was developed and evaluated as a diagnostic test, because confirmation of M. synoviae infection by culture presents several practical challenges (**Kleven *et al.* 2008**, **Garcia *et al.*, 1996**, **Lauerman, 1998**, **Feberwee *et al.*, 2005**). M. synoviae is fastidious organism with unique medium requirements; it requires 4 days up to 5 days for growth (**Kleven *et al.*, 2008**), even up to 28 days of incubation (**Feberwee *et al.*, 2005**), and initial cultures commonly contain other mycoplasmas, such as M. gallinarum and M. gallinaceum (**Kleven 1998**) and the results showed that *Mycoplasma synoviae* was confirmed using PCR targeting 16s rRNA gene. Polymerase chain Reaction using primers targeting 16s RNA and specific gene was performed, DNA extracted from samples resulted in five positive sample for preparation of broth out of 17 examined samples with a percentage of (29.4%), three of them were positive for PCR using primers targeting 16s RNA gene (figure1), these results were nearly similar as prevalence rate was reported in Zimbabwe (<33%) **(Kelly *et al.*, 1994)** and Uberlandia (26%) (**Pereira**, **2005**). On the other hand, the higher prevalence was reported in backyard chickens in Benin (62%) (**Chrysostome *et al.*, 1995**), Botswana (57.8%) (**Mushi et *al.*, 1999**), South Africa (63%) (**Thekisoe *et al.*, 2003**), Ecuador (73%) (**Hernandez-Divers *et al.*, 2006**), Bangladesh (58.9%) (**Sikder *et al.*, 2005**), Argentina (68.6%) (**Xavier *et al.*, 2011**), Ethiopia (67.7%) (**Chaka *et al.*, 2012**), and Brazil (53.3%) (**Silva *et al.*, 2015**). **Buchala *et al.*** (**2006**) and **Haesendonck *et al.*** (**2014**) demonstrated a very high prevalence of *M. synoviae* with 100% and 96.4% of flocks, respectively, being positive, suggesting that this group of birds might act as a potential reservoir for Mycoplasma. In Egypt **El shafey *et al.*, (2016)** stated that PCR was performed to DNA extracted from chicken samples resulted in 25 positive sample out of 103 (24.2%), from which the percentage was 30% from bumbled foot exudate, 45% from synovial fluid of arthritic joint so these results supported our results. In this study two published primer sets were used for the specific detection of genus and species of *M. synoviae* by amplification with specific primers as described by **Zhao**, **S. and R. Yamamoto**, (**1993**) as showed in table (2) results revealed two positive field sample at 1000bp figure (2). MS specific gene was also successfully sequenced, published in Gen Bank were selected as Reference sequences for M.S showing arthiritis in Egypt. In the backyard chickens were neither given any immunizations nor afforded treatments, which make them intrinsically sensitive to mycoplasma infection Phylogenetic analysis of DNA sequence of M.S detected (M.synoviae-SB-YardDG.019) revealed high homology with the M.S strains M.synoviae-Eis2-16 with a percentage of identity 99.05% and with a percentage of identity 99.47% with M.synoviae-ATCC-5204. (Figure 3 &4). These results in the backyard chickens revealed that these chickens were neither given any immunizations nor afforded treatment. A sequence results were reported by **Meghan *et al.,*** (**2015**), **Amer *et al.,*** (**2019)** who make molecular identification of M.S from breeder chicken flock showing arthiritis belonged to mycoplasma infection. Haematological investigation of diseased birds were applied, results showed the -difference in total &differential leukocyte count ,total protein, Creatinine, Uric acid, AST and ALT between clinically diseased birds positive for M.S specific strains (SB-YardDG.019) and other clinically diseased birds negative for this specific strains. Results revealed increased total leukocyte count in clinically diseased birds positive for M.S specific strains (SB-YardDG.019) from 31.000 ± 1.500 to 47.300 ± 4.000 (10^3/mm^3), increased Neutrophils from 6.250 ± 300 to 12.200 ± 100 10^3/mm^3) than in diseased birds negative for M.S specific strains, lymphopenia as lymphocyte decreased from 29.040±269 to 13.000±

500 (10^3/mm^3), eosinopenia as eosinophils decreased in these diseased birds to 826±50 than 1.145±150 (10^3/mm^3), Basophils decreased from 130±15 to 40± 50 (10^3/mm^3) and monocytosis as monocyte increased from 1.500±117 to 3.150±200 (10^3/mm^3) in these birds. Also total protein was increased from 5.69±0.50 to 7.34±0.50 g/dl in diseased birds with M.S specific strains, Creatinine, Uric acid, AST and ALT were also increased as showed in table (4). Results revealed that ,diseased birds with M.S which diagnosed by PCR revealed heterophilia, lymphopenia, monocytosis, eosinopenia and basopenia and increased total protein in diseased birds **Branton *et al.*, (1997)** stated that chicken infected-with-M.SWVU-1853 strain revealed-heterophilia lymphopenia, monocytosis, basopenia and eosinopenia in acute infection.

Conclusion: In conclusion, our findings seem to be consistent with many studies which suggest that eradication program for Mycoplasma in poultry especially in backyard groups is essential for control its transmission and reducing its threats for poultry health. Also molecular method as PCR and specific gene Sequencing are considered essential in diagnosis of M. Synoviae.

References

Adair, B.M.; Burns, K.; McNulty, M.S. and Todd, D. (1990). A study of ELISA systems incorporating pooled viral and mycoplasma antigen preparations for antibody screening of chicken sera. Avian Pathology 19: 263-278.

Amer, M.M.; Mekky, H.M. and Fedawy, H.S. (2019). molecular identification of M.S from breeder chicken flock showing arthiritis in Egypt. Vet. World, 12(4): 535-541.

Branton, S.L.; May, J.D.; Berry, D.L. and William, R.M. (1997). Various blood parameters in commercial hens acutely and chronically infected with mycoplasma gallisepticum and mycoplasma synoviae. Avian Dises. 41(3): 540-547.

Catania, **S**., **Bilato**, **D**., **Gobbo**, **F**., **Granato**, **A**., **Terregino**, **C**., **Iob**, **L**. **and Nicholas**, **R**.**A**. (**2010**): Treatment of eggshell abnormalities and reduced egg production caused by Mycoplasma synoviae infection. Avian Dis., 54(2): 961-964. 8. Jeon, E.O., Kim, J.N.,

Chaka, H.; F. Goutard; S.P. Bisschop and P.N. Thompson (2012). Sero prevalence of Newcastle disease and other infectious diseases in backyard chickens at markets in Eastern Shewa zone, Ethiopia. *Poultry science,* 91, (4): 862-869.

Chrysostome, C.; J. Bell; F. Demey and A. Verhulst (1995). Sero prevalences to three diseases in village chickens in Benin. *Preventive veterinary medicine,* 22, (4): 257-261.

Doumas, B.T.; D.D. Bayse; R.J. Carter; T. Peters and R. Schaffer, (1981). A candidate reference method for determination of total protein in serum. I. Development and validation. *Clinical Chemistry,* 27, (10) 1642-1650.

Doumas, B.T.; D.D. Bayse; R.J. Carter; T. Peters and R. Schaffer (1981). A candidate reference method for determination of total protein in serum. I. Development and validation. *Clinical Chemistry,* 27, (10) 1642-1650.

Dufour-Gesbert, F.; A. Dheilly; C. Marois and I. Kempf (2006). Epidemiological study on Mycoplasma synoviae infection in layers. *Veterinary microbiology,* 114, (1-2) 148-154.

El shafey, D.Y.; S.I. Eissa; M.A. Dardeer; E.E. Abd El-Aziz and A.M. Hassan (2016). Prevalence of mycoplasma synoviae infection in broilers and layers with special emphasis to in vitro antimicrobial sensitivity. *IJAR,* 4, (3): 1336-1343.

Erno, H. and L. Stipkovits, (1973). Bovine mycoplasmas: cultural and biochemical studies. I. *Acta Veterinaria Scandinavica,* 14, (3) 436.

Feberwee, A.; Mekkes, D.; De Wit, J.; Hartman, E. and Pijpers, A. (2005). Comparison of culture, PCR and different serologic tests for detection of mycoplasma gallisepticum and mycoplasma synoviae infection. Avian diseases 49, 260 – 268.

Ferguson-Noel, **N**. **and Noormohammadi**, **A**.**H**. (**2013**) Mycoplasma synoviae infection. In: Swayne, D.E., Glisson, J.R., McDougald, L.R., Nolan, L.K., Suarez, D.L. and Nair, V., editors. Diseases of Poultry.

13th ed. Blackwell Publishing, United Status. p900-910.

Frey, M.L.; Hanson, R.P. and Anderson, D.R. (1968). A medium for the isolation of avian mycoplasmas. AmJ Vet Res 29, 2163-2171.

Garcia, M.; Jackwood, M.W.; Head, M.; Levisohn, S. and Kleven, S.H. (1996). Use of species-specific oligonucleotide probes to detect Mycoplasma gallisepticum, M. synoviae and M. iowae PCR amplification products. Journal of Veterinary Diagnosis Investigation 8: 56-63.

Haesendonck, R.; M. Verlinden; G. Devos; T. Michiels; P. Butaye; F. Haesebrouck; F. Pasmans and A. Martel (2014). High seroprevalence of respiratory pathogens in hobby poultry. *Avian diseases,* 58, (4): 623-627.

Hall, T.A. Bio Edit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT Nucl Acids Symp Ser. 1999; 41: 95-98.

Hernandez-Divers, S.M.; P. Villegas; F. Prieto; J.C. Unda; N. Stedman; B. Ritchie; R. Carroll and S.J. Hernandez-Divers, (2006). A survey of selected avian pathogens of backyard poultry in Northwestern Ecuador. Journal of Avian Medicine and Surgery, 20, (3): 147-158.

Higgins, D.G. and Sharp, P.M. (1989). Fast and sensitive multiple sequence alignments on a microcomputer. Computer Applications in the Biosciences. 1989; 5: 151-153.

Jain, **N.C. (1986).** Schalm Veterinary Hematology. Lea & Febiger, Philadelphia PA.

Kelly, L.M. and L.C. Alworth (2013). Techniques for collecting blood from the domestic chicken. *Lab animal,* 42, (10): 359.

Kelly, P.J.; D. Chitauro; C. Rohde; J. Rukwava; A. Majok; F. Davelaar and P.R. Mason, (1994). Diseases and management of backyard chicken flocks in Chitungwiza, Zimbabwe. *Avian diseases*, 626-629.

Kierończyk, B., Rawski, M., Józefiak, D. and Świątkiewicz, S. (2017) Infectious and non-infectious factors associated with leg disorders in poultry a review. Ann. Anim. Sci., 17(3): 645-669.

Kleven, S.H. (1998). Mycoplasmas in the etiology of multifactorial respiratory disease. FtJult Science 77: 1146-1149.

Kleven, S.H. (2008). Mycoplasma synoviae infection. In: Saif YM, Barnes HJ, Fadly AM, et al, eds. Diseases of Poultry. Ames, Iowa: Iowa State University Press,756-766.

Kumar S, Tamura K, Nei M. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform.2004;5:150-63.

Lauerman, L.H., (1998). Mycoplasma PCR assays. In: Lauerman, L.H. (Ed.), Nucleic Acid Amplification Assays for Diagnosis of Animal Diseases. M. gallisepticum and Mycoplasma synoviae infections. Avian Diseases 49: 260-268.

Lee, H.R., Koo, B.S., Min, K.C., Han, M.S., Lee, S.B., Bae, Y.J., Mo, J.S., Cho, S.H., Lee, C.H. and Mo, I.P. (2014) Eggshell apex abnormalities associated with Mycoplasma synoviae infection in layers. J. Vet. Sci., 15(4): 579-582.

Majid, E.; Maryam, A.; Mehrdad, S.B. and Mojtaba, A. (2015). Diagnosis of Mycoplasma synoviae by plate agglutination and ELISA tests in commercial broiler chickens. Journal of Vet. Res. volume 19(9): 593-597, 2015.

Meghan, A.M.; Gerald, F.K.; Anthony, F.B.; Dina, L.M. and Daniel, R.B. (2015). Complete Genome sequence of Mycoplasma Synoviae strain WVU1853 Genome Announce 2015 May – Jun. 3(3).

Mushi, E.; M. Binta; R. Chabo; M. Mathaio and R. Ndebele (1999). Detection of Mycoplasma gallisepticum and Mycoplasma synoviae antibodies in the sera of indigenous chickens by rapid serum agglutination test at Mmopane, Gaborone, Botswana. *Onderstepoort Journal of Veterinary Research,* 66, (4) 333-334.

OIE (2008). Manual of Diagnostic tests and vaccine for Terrestrial Animals (Mammals, Birds and Bees), Avian Mycoplasmosis (Mycoplasma gallisepticum M.Synoviae).6 thEdition, OIE. pages 482-496.

Pereira, M. (2005). Prevalência de anticorpos contra Mycoplasma gallisepticum, Mycoplasma synoviae e Salmonella pullorum e identificação bacteriológica de Salmonella spp. em galinhas “caipiras” no município de

Uberlândia [Monografia]. *Uberlândia (MG): Universidade Federal de Uberlândia*.

Ranck, M.F.; V. Schmidt; H.C. Philipp; M. Voss; J. Kacza; A. Richter; K. Fehlhaber and M.E. Krautwald-Junghanns, (2010). Mycoplasma synoviae-associated egg-pole shell defects in laying hens. *Berliner und Munchener tierarztliche Wochenschrift,* 123, (3-4) 111-118.

Reitman, S. and S. Frankel, (1957). A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *American journal of clinical pathology,* 28, (1) 56-63.

Saadia, N.; Aboukhalid, R.; Azzam, F. and Rahmatallah, N. (2014). Detection of Mycoplasma synoviae infection in broiler breeder farms of Morocco using serological Assays and Real time pcr. Life Science journal, 8: 815-821.

Sanders, G.T.; Pasman, A.J. and Hoek, F. (1980). Determination of uric acid with uricase and peroxidase. Clin. chim, Acta. 101: 299-303.

Sikder, A.; M. Islam; M.M. Rahman and M. Rahman, (2005). Seroprevalence of Salmonella and Mycoplasma gallisepticum infection in the six model breeder poultry farms at Patuakhali district in Bangladesh. *International Journal of Poultry Science,* 4, (11) 905-910.

Silva, C.B.C.D.; W.D.F. Chagas; R.F.D. Santos; L.R. Gomes; M.R. Ganda and A.M.C. Lima (2015). Seroprevalence of salmonella and mycoplasma in commercial broilers, backyard chickens, and spent hens in the region of triângulo mineiro, State of Minas Gerais, Brazil. *Revista Brasileira de Ciência Avícola,* 17, (1) 57-62.

Stanley, W.A., Hofacre, C.L., Speksnijder, G., Kleven, S.H. and Aggrey, S.E. (2001) Monitoring Mycoplasma gallisepticum and Mycoplasma synoviae infection in breeder chickens after treatment with enrofloxacin. Avian Dis., 45(2): 534-539.

Sumitha, P. and Sukumar, K. (2017) Incidence of Mycoplasma synoviae induced eggshell apex abnormality in desi chicken. Indian Vet. J., 94(5): 36-37.

Sun, K.S.; Lin, X.; Chen, F.; Wang, D.A.; Lu, J.P.; Qin, J.P. and Luo, T.R. (2017). Epidemilogical investigation of Mycoplasma synoviae in native chicken breeds in China. BMC vet, Res. 2017; 13: 115.

Thekisoe, M.; P. Mbati and S. Bisschop (2003). Diseases of free-ranging chickens in the Qwa-Qwa District of the northeastern Free State province of South Africa. *Journal of the South African Veterinary Association,* 74, (1) 14-16.

Thompson, J.D.; Higgins, D.G.; Gibson, T.J. and Clustal, W. (1994). Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positionspecific gap penalties and weight matrix choice. Nucleic Acids Research. 1994; 22: 4673-80.

Zhao, S. and Yamamoto, R. (1993a). Detection of Mycoplasmameleagridis by polymerase chain reaction. Veterinary Microbiolog-Volume 36, Issues 1–2, July, Pages 91-97.