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DIAGNOSIS OF *MYCOPLASMA GALLISEPTICUM* INFECTION BY RECENT TECHNIQUE IN LAYING CHICKEN FLOCKS

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SUMMARY

Different methods were used for diagnosis of mycoplasma infection in layers farm at different ages. A total of one hundred and sixty four samples were collected as follow: (30) nasal swabs, 84 pharyngeal swab, 50, lung, air sac &trachea, and 96 serum samples. The primary isolation and identification revealed that 60 isolates *Mycoplasma* (M), from total of 164 organs samples (25/50isolates from lung, trachea and air sac (50%) at 3weeks of age, and 5/34 from pharyngeal swabs (14.7%) ,and 3/15 from nasal swabs (20%) at 13 weeks of age.

Also MG isolates20/50 from pharyngeal swabs (40%), and 7/15 from nasal swabs (46.7) at 30 weeks of age. Antigenic and genetic variability between MG field isolates and reference MG strain (R) were studied by using polymerase chain reaction test (PCR) with primers complementary to the 16s rRNAgens was used to diagnose *Mycoplasma gallisepticum* field and reference strains which amplified by the oligonucleotide primers and gave acharacteristic fragment of 330bp.

Diagnosis of MG by using molecular techniques is more specific and more rapid than conventional procedure. Also RAPD-PCR test could successfully differentiate between different MG strains.

We concluded that individual strains of MG are genetically quite unique and this test is reliable method for diagnosis and differentiation of *Mycoplasma gallisepticum* strains and can play an important role in understanding the epidemiology and spread of the disease, Serologic tests as Serum plate

agglutination (SPA) and ELISA can indicate seroconversion; however, they could be used more satisfactorily for flock screening. Seven antibiotics were studied for their protection and treatment of mycoplasma infection in laying birds. It was better to use norfloxacin, enrofloxacin, ciprofloxacin ,& Erythromycin to control MG .

INTRODUCTION

Mycoplasma gallisepticum (MG) is an important avian pathogen causing economic losses to the poultry industry and most significantly impacting the egg layer industry. In breeders and layers, the diseases cause tremendous drop in egg production, increase in embryo mortality, leading to infected eggs and infected progeny flocks (Cassell et al., 1985, Bradbury, 2001 and Ley, 2003). Also Yoder, (1991), Zeinab, (2001) and Butcher, (2004), reported that MG continued to be a major problem in multi-age commercial layers responsible for economic losses due to decreased egg production and hatchability, increase embryo death, downgrading and condemnation of carcasses, and decreased feed efficiency.

The control of MG infection has included maintaining flocks free of the pathogenic organisms, applying good biosecurity practices, using of antibiotics in both prophylactic and therapeutic regimes which was faced by antibiotic resistance and vaccination whether with low virulence MG F strain vaccine or inactivated MG bacterin which can be useful long –term solution, especially on multi-age commercial egg production sites (Kleven, 1990,2008, Zonder et al, 1997, and Branton et al,1999).

Diagnosis of mycoplasma infection is based mainly on clinical signs ,isolation of organisms, serological tests such as Serum Plate Agglutination (SPA), Heamagglutination Inhibition (HI) and ELISA tests which have been used routinely .Problems of low sensitivity ,cross reaction , and non specific reactions have been encountered with RSA and HI tests ((Mallinson and Rosenstein, 1976) .Recently PCR techniques for MG diagnosis have been applyed as multiplex PCR and PCR-RFLP developed for mycoplasmas detection and identification (Kiss,et al.,(1997).In addition to PCR with arbitrary primers and Random Amplify polymorphic DNA analysis (RAPD). Lauerman,et al.,(1993), Bradbury,et al.,(2001) and Fan,et al.,(1995) were used for strains differentiation and very useful for epidemic study of disease.

Mycoplasma gallisepticum (MG) has shown sensitivity in vitro and in vivo to several

antimicrobials including; Macrolides, Tetracyclines, Fluroquinolons and others (Bradbury, et al., 1994, El-shabiny et al.,1997. Jordan et al., 1998 and Wang et al., 2001), and using of antimicrobials remains the most common means of controlling of MG and MS infections although antimicrobial resistance has been reported and a comparative overview described by Valks and Burch (2001).The existence and the persistence of MG in commercial poultry farms suggested that efforts of eradication were not successful; therefore, limiting losses is the primary objective (Gary, 2004). To achieve successful treatment of flocks with antimicrobials, it is necessary to asses the sensitivity of mycoplasmas present in the flocks (Burch and Stipkovits, 1994).

The present study aimed to diagnose MG infection in laying chicken flocks, from different farms by conventional culturing procedure, specific PCR test and comparing between MG isolates (field, vaccinal and reference strains) by using random amplified polymorphic DNA (RAPD) method, also by using RSA and ELISA tests. In addition, to study differentiation and the efficacy of some antibiotics against the isolated strains by growth inhibitions test.

MATERIAL AND METHOD

1-Samples

In this study a total of two hundred and sixty samples were collected from laying flocks at different ages ,and from different layers farms as follow: (30), nasal swabs, (84), pharyngeal swabs, (96) serum samples. ,{from living birds},and (50) trachea, lungs and air sacs,{from freshly dead birds} . The examined samples were from birds with and without clinical signs.

2- Reference MG strains:

Reference strain used in this study was (R, strain) pathogenic MG strain, from Mycoplasma Dept., Animal Health Research Institute (AHRI), DokkI, Giza Egypt.

3-Isolation and identification of MG:

-Liquid and solid media used for isolation and propagation of *mycoplasma* were prepared as described by Frey et al., (1968).

-Genus determination and biochemical characterization were carried out as described by (Erno and Stipkovits, (1973).

4- Serological identification:

a-Serum Plate Agglutination (SPA): according to Kleven and Yoder (1989).

b-Enzyme Linked Immunosorbent Assay (ELISA): according Higgins and Whithear (1986) .

5-Polymerase Chain Reaction (PCR): as described by (Kempf et al., 1993):

1-Extraction of chromosomal DNA (Fan et al., 1995): A five ml quantity of overnight culture from each Mycoplasma isolate was centrifuged in a micro-centrifuge at 13000 rpm. for 3 minutes. The cell pellets were washed twice in 100 µl of 150 mM phosphate- buffered saline (PBS, pH 7.2) and suspended in 25 µl PBS. The cell suspension was heated directly at 100 °C for 10 minutes in a heat block and collected on ice for 10 minutes. Finally, the cell suspension was centrifuged for 3 minuets, and chromosomal DNA was collected and stored at 4°C.

2-Primer selection(Kempf et al.,1993):

Two oligoneucleotide primers were selected for the detection of MG. The sequence of primer (1) was: 5\- TAA CTA TCG CAT GAG AAT AAC-3\. The sequence of primer (2) was 5\-GTT ACT TAT TCA AAT GGT ACA G-3\. The primer was locally prepared using 392 DNA/RNA synthesizers (Applied Biosystems) in Mycoplasma Department, AHRI, Dokki, Giza, Egypt.

3-Procedure for DNA amplification:

The reaction mixture (total volume 100 µl) was 10 µl of 10 X reaction buffer (Promega), 3 µl 25 mM MgCl2, 12 µl of 10 mM of each dNTP mixture (Sigma), 2 µl primer (containing 400 ng of each left and right primer), 2 µl DNA template (containing 40 ng DNA), 0.5 µl (2 units) of taq DNA polymerase (Promega), and complete the mixture with 80.5 µl distilled water. DNA amplification was carried out in a PTC- 100 programmable thermal controller (M.J. Research Inc.).The amplification was performed by heating the samples for 5 minutes at 97 °C then, using thirty cycles of denaturation for 1 minute at 94 °C, annealing for 1 minute at 55 °C and extension for 1 minute at 72 °C with the exception that final extension step was held for 10 min. The analysis of PCR amplified products was done by using ten µl of the amplified PCR product, mixed with 2 µl loading buffer and electrophoresed through 0.8% agarose gel and DNA was visualized by UV flourescence after ethidium bromide staining, and then photographed. Image analysis was made by Image Quant TL-2003 software of Amersham Bioscience.

4-Arbitrary primer:

The oligonucleotide primer used in this study was M16SPCR5. The M16sPCR5`, primer was based on the sequence of 16SrRNA of MG (Gene Bank Acc. No. M22441). Table 2 lists the sequence, size, guanine plus cytosine content, and melting temperature of this primer.

5-Amplification conditions:

The reaction mixture (total volume 100 ml) was 10ml of 10 x reaction buffer (promega), 8 micro-liter, 25mM MgCL2 ,16 micro-liter of 10

mM of each nucleotide (dATP, dCTP, dGPT and dTTP, sigma, 2 µl primer (containing 400 ng of each left and right primer), 5 µl DNA template (containing 40 ng DNA),0.5 µl (2units) of tag DNA polymerase (promega) and complete the mixture with 59.5 ml distilled water. PCR was performed on a PTC-100 programmable thermal cycler controller (M.J. Research Inc.) The amplification conditions was three cycles of 94°C for 15 seconds .28°C for 2 minutes and 74 °C for 3 minutes and for 35 cycles of 94 °C for 15 sec, 45°C for 2 minutes and 74 °C for 3 minutes.

Table (1): Base sequence and size of the arbitrary primer used

Primer	base sequence	No. of bases	G+C %	Melting point
M16sPCR5'	5'AGGCAGCAGTAGGGAAT3'	17	52.9	43.7°C

G + C =Guanine plus cytosine.
RAPD patterns analysis was performed by Image analysis by Image Quant TL-2003 software of Amersham Bioscience. Each RAPD analysis gel was standardized by comparison of *Mycoplasma gallisepticum* unknown isolates to reference strains. Isolates were considered identical when major differences could not be visualized

Table (2) Source of MG reference strains and field isolates used.

Strain or isolate	Isolated from	Source
R	Chicken respiratory tract	Reference strain
Field	Trachea/lung/air sac	Field isolate
Field	Pharyngeal swabs	Field isolate
Field	Nasal swabs	Field isolate

6- Antibiotic sensitivity test: according to (Clyde 1964). Seven different antibiotic discs including: enrofloxacin, lincospectin, ciprofloxacin, norfloxacin, spectinomycin, erythromycin and tiamulin, were examined against field isolates of mycoplasma.

RESULTS

Results of mycoplasma isolation:

Some of examined samples (organs) showed air sacculitis, pneumonia, andtrachitis. The primary isolation and biochemical characterization of Mycoplasma isolates from different collected samples yielded (60 out of 164) Mycoplasma isolates (digitonin positive 36.6%), and (16) Acholeplasma (digitonin negative 9.8%)as shown in table (3)

Table (3): Mycoplasma isolation from different samples and biochemical characterization.

Bird age	Site of isolation	Sample No.	Positive No.	Biochemical characterization				Suspected Mycoplasma
				D	G	A	F&S	
3weeks	Trachea Lungs Air sac	50	25	23 -	23 -	- 2	- 2	*M.gallisepticum* *M.gallinarum*
13weeks	Pharyngeal& nasal swabs	34 15	5 3	5 3	5 3	- -	-	*M.gallisepticum*
30weeks	Pharyngeal& nasal swabs	50 15	20 7	20 7	20 7	- -	-	*M.gallisepticum*

D=No. of digitonin positive G= No. of Glucose positive
A=No.of Arginine possitive F&S= Film and spot formation

Results of PCR test.

Polymerase chain reaction (PCR) test was carried out on mycoplasma field isolates.

The PCR results detected the presence of a characteristic common band at 330 bp in all isolated and the reference strains(R) fig. (1) & (2).

PCR identificatio of the obtained isolates recorded the following results, (23) MG isolates from respiratory organs and air sac (46%), (8) MG isolates from pharyngeal And nasal swabs at 13 weeks of age (16.3%) and 27 MG isolates from swabs at 30 weeks of age (41.5%) as in table (4).

Table (4): identification of Mycoplasma gallisepticum positive isolates by specific PCR.

Bird age	Number of examined samples	Number of positive MG	Percentage of MG
3 weeks	50	23	46%
13 weeks	49	8	16.3%
30 weeks	65	27	41.5%

Results of Random Amplified Polymorphic DNA (RAPD) analysis for the typing of *M. gallisepticum* isolated from different organs of birds.

Our results demonstrate that, the use of primer M16SPCR5\ for the PCR amplification of different *Mycoplasma gallisepticum* DNAs from different organs at different ages resulted in reproducible DNA fragment patterns each unique to a particular strain, although many fragments appeared common to several strains, the patterns were qualitatively sufficient accurate strain differentiation .

As shown in Fig. (3) and table (5) by using this primer, reference pathogenic strains R yielding more bands than other field isolates.

Table (6) declared that *M. gallisepticum* field isolates from birds were shown to give bands varied from 245 - 1220 bp. Two isolates shared in one band with the reference strain at 358 bp. All isolates shared with each other in two bands at 392 bp and 491 bp. Two isolates shared in three bands at 420, 943 and 1100 bp while the other two isolates shared at 358,570, 1150, and 1220.

From the mentioned results before, there were some intra species heterogeneity between the field isolates of *M. gallisepticum* isolated from different organs.

Table (5): Common and characteristic bands among *M.* gallisepticum field isolates in comparison with the reference R strain

	M. gallisepticum reference strain		M. gallisepticum field isolate		
	(R)	1	2	5	6
No. of bands	5	7	6	8	7
Common bands	1	5	5	6	6
Characteristic DNA bands	588 693 1450 1885	245 913	269	431 750	452

Table (6) RAPD-PCR analysis of Mycoplasma gallisepticum reference strains and field isolates from layers.

Marker	Lane 2	Lane3	Lane4	Lane5	Lane6	Lane7	Lane8
100	358	245	269			358	358
200		392	392			392	392
300	588	420	420			431	452
400	692	491	491			491	491
500	1450	912				570	570
600	1885	943	943			750	
700		1100	1100			1150	1150
800						1220	1220
900							
1000							
1100							

Analysis was done by 1D LabImage program.

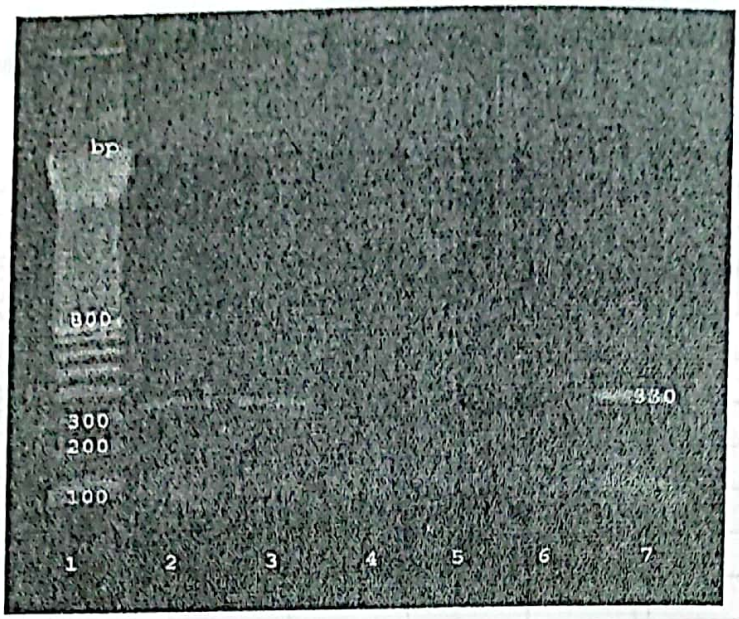

Fig. (1): Agrose gel electrophoresis of the PCR product of amplified regions in the 16srRNA genes of MG strains.
Lane 1: 100bp DNA Ladder.
Lane 2: Isolated DNAfrom nasal swabs.
Lane 3: Isolated DNAfrom Pharyngeal swabs.
Lane 4, 5: negative swabs
Lane 6: Negative control
Lane 7: positive control

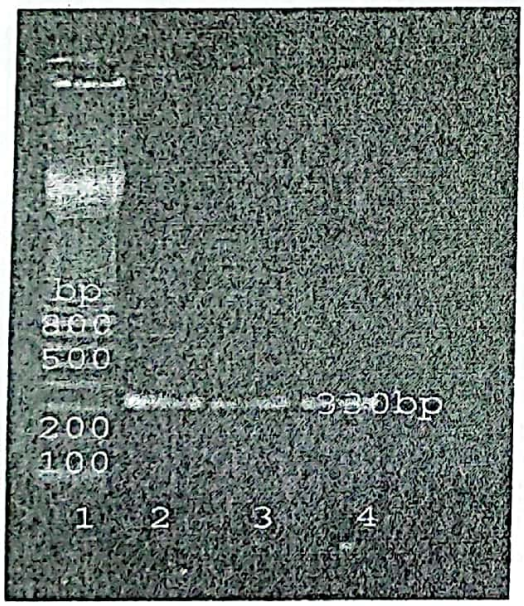

Fig. (2) Agrose gel electrophoresis of the PCR product of amplified regions in the 16srRNA genes of MG strains.

Lane 1:100 bp DNA Ladder.

Lane 2: MG control positive.

Lane 3: isolated DNA from lungs.

Lane 4: isolated DNA from trachea &air sacs

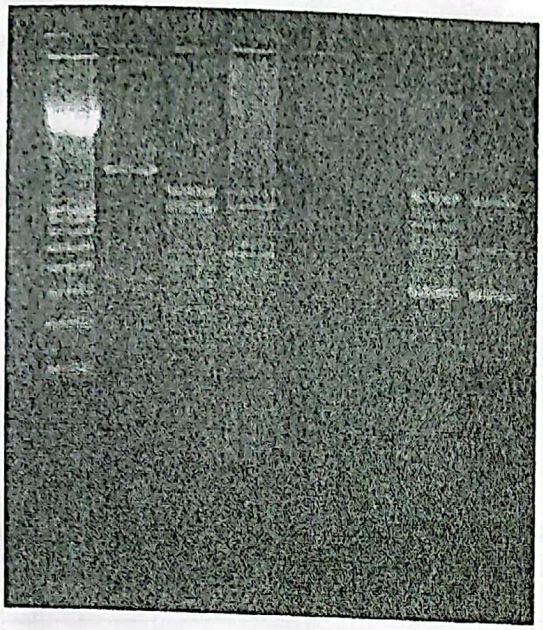

Fig.(3): Electrophoretic analysis of RAPD-PCR Patterns mycoplasma gallisepticum. Reference and field strains.

Lane 1: 100 bp marker.

Lane 2: R strain(reference strain).

Lane 3:-Field isolates from trachea.

Lane 4:- Field isolates from lungs & air sacs

Lane 5&6: negative field isolates.

Lane 7: Field isolates from nasal swabs.

Lane 8: Field isolates from Pharyngeal swabs.

Results of SPA and ELISA tests on sera of birds.

SPA test and ELISA were used for the detection of immune response. The positive results of SPA and ELISA tests increased gradually by aging. Average of optical density (O D) values for serum samples = 1.790 -0.162 Negative =0.162Positive = 1.800 Table (7) shows that agglutinins were detected in examined sera. There are 51/96 positive sera for MG antibodies by SPA test (53%). And 48/96positive by EELISA test (50%).

Table (7) Results of SPA and ELISA tests on sera of birds (no. of positive / no. of examined)

	SPA test						ELISA					
Week / Sample	13	20	25	30	35	40	13	20	25	30	35	40
Sera*.	5/16	7/16	9/16	9/16	10/16	11/16	4/16	6/16	8/16	9/16	10/16	11/16

Total serum samples are 96. Sera*= No.of positive / No.of examined sera.

Results of antibiotic sensitivity test.

In vitro sensitivity of mycoplasma isolates showed varying degree of inhibition to antimycoplasmal agents, the isolates were highly sensitive to norfloxacin, enrofloxacin, ciprofloxacin, Erythromycin and moderetly sensetive to, lincospectin, Spectinomycin, Tiamulin as shown in Table (7).

Table (7) Sensitivity of isolated mycoplasma from different samples against different antimicrobials discs.

Antibiotic disc	Disc conc. (µg)	Standard zone of inhibition (≥)mm	No. of examined strains	Sensitive strains	
				No.	Percent
Lincospectin	15	19	15	10/15	66.7
Norfloxacin	10	29	15	13/15	86.7
Enrofloxacin (ENR)	5	30	15	13/15	86.7
Ciprofloxacin (CF)	5	29	15	13/15	86.7
Tiamulin	100	21	15	9/15	60
Spectinomycin	10	19	15	10/15	66.7
Erythromycin	10	28	15	12/15	80

DISCUSSION

The present study aimed to diagnose MG infection in laying chicken flocks. The results of Isolation and identification of the organism is the "gold standard" for the diagnosis of mycoplasma infections. Procedures for isolation and identification, including

formulations of commonly used media, are available. However, mycoplasmas are slow growing organisms and are commonly overgrown by commensals such as M. gallinarum (Kleven, 2008).

Our results demonstrated that using primer M16SPCR5 used to amplify genomic DNAs of *Mycoplasma gallisepticum* isolates resulted in reproducible fragment patterns which could be used to differentiate and group strains of MG.

Pathogenic strain R could be distinguished with this primer, they shard a similar banding pattern which support a previous report of the clustering of these strains -Yogev,et, al.(1989). This also agrees with Geary et, al. (1994).

Reliable methods for the differentiation of *mycoplasma gallisepticum* strains play a pivotal role in understanding the epidemiology and spread of the disease because they generate the information necessary to identify and track new outbreaks. Ideally, methods of strain differentiation must have high enough discriminatory power to clearly differentiate unrelated strains, as well as to demonstrate the relationship of isolates from individuals infected through the same source.

Concerning immune –response: the highest antibody response was that detected by SPA test followed by ELISA. This data agree with Yoder et al., (1984), Refai et al., (1993),and Abd El-Motalib and Kleven (1993).

Also, Talkington et, al. (1985) concluded that ELIZA test is sensitive and can replace SPA and HI tests for detecting MG antibody.

Results of tested antibiotics showed good effect against the isolated strains, and quinolones group give the best bactericidal effect on mycoplasma isolates ,this results agree with Sabry, (2004) .

CONCLUSION

In commercial layers, losses can be reduced through bio-security programs and effective use of vaccines and effective antibiotic.

Serologic tests can indicate seroconversion; however, they could be used more satisfactorily for flock screening. Diagnosis of MG by using molecular techniques is more specific and more rapid than isolated procedure. Also RAPD-PCR test could successfully differentiate between different and virulent MG strain

We concluded that individual strains of MG are genetically quite unique and this test is reliable method for the differentiation of *Mycoplasma gallisepticum* strains and can

play an important role in understanding the epidemiology and spread of the disease.

For protection and treatment of mycoplasma infection in layers bird, it is bbetter to use norfloxacin,enrofloxacin ,ciprofloxacin,&erythromycin to control MG as these were highly

Active against MG.

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تشخيص عدوى الميكوبلازما جاليسبتيكم فى مزارع البياض بالطرق الحديثة

زينب رشدى ، دينا الشافعى ، عبد السعيد ، فادية عبد الحميد

قسم الميكوبلازما معهد بحوث صحة الحيوان ، الدقى

تم دراسة طرق مختلفة لتشخيص عدوى الميكوبلازما فى مزارع البياض من أعما ر مختلفة . تم تجميع عدد (30) مسحة أنفية و(84) مسحة من الحنجرة و(50) عينة من الرئة ـالقصبة الهوائية ـالاكياس الهوائية و(96) عينة سيرم .تم حيث تم عزل و تصنيف 60معزولة للميكوبلازما جاليسبتيكم من أجمالى 164 عينة, بواقع 50/25 معزولة من أنسجة الرئة والقصبة الهوائية والاكياس الهوائية عمر 3 أسابيع بنسبة 50% و عدد 5/ 34 من مسحات الحنجرة بنسبة (7,14%) و15/3 من مسحات الأنف بنسبة (20%) عند عمر 13 أسبوع وكذلك تم عزل عدد 50/20 من الميكوبلازما بنسة (40%) من مسحات الحنجرة وعدد 15/7 من مسحات الأنف بنسبة (7,46%) عند عمر 30 أسبوع . تم ايضاح الاختلافات الجينية بين بعض السلالات الحقلية للميكوبلازما جاليسبتكم و مقارنتها بسلالة مرجعية با ستخدام أختبار تفاعل انزيم البلمرة المتسلسل لتاكيد التصنيف حيث أعطى حزمة مميزة عند 330 قاعدة مزدوجة. بدراسة اختلاف الحمض النووى بين سلالات الميكوبلازما جاليسبتكم المعزولة و المرجعية باختبار تكبير الحمض النووى العشوائى تبين وجود اختلافات مميزة بين جميع السلالات بالرغم من و جود حزم مشتركة بينهم و تبين أن الاختبار يمكن استخدامة فى التشخيص السريع والدقيق للا صابة بالميكوبلازما . أ مكن الاستدلال على التحول المصلى باستعمال اختبارى الاليزا والتلازن فى التشخيص ولكن من الافضل استعمالهما فى فحص القطيع.كذلك تم أختبار سبعة مضادات حيوية لمعرفة حساسية معزولات الميكوبلازما جاليسبتكم و أعطى النورفلوكسين والانروفلوكسين أفضل نتائج. وبهذا يتبين أن أ ختبار تفاعل أنزيم البلمرة المتسلسل أسرع وادق طرق التشخيص .