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SITUATION OF MYCOPLASMA INFECTIONS AMONG CHICKENS IN UPPER EGYPT WITH EVALUATION OF DIFFERENT DIAGNOSTIC TECHNIQUES

(With 7 Tables)

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وضع الإصابة بالميكوبلازما بين الدجاج بصعيد مصر مع تقييم الإختبارات التشخيصية المختلفة

مصطفى سيف الدين

أظهرت الدراسة أن معدل الإصابة بالميكويلازما جاليسبتكم والميكويلازما سينوفى بين قطعان الدجاج المختلفة فى صعيد مصر كان بنسبة ١٠٠٪ فى قطعان التسمين، ٦٦٪ فى قطعان إنتاج البيض و ٤٠٪ فى أمهات التسمين. وتم تقييم كل من الإختبارات السيرولوجية المختلفة ، الزرع البكتيريولوجى وسلسلة تفاعل البلمرة لتمييز الحمض النووى فى تشخيص الميكوبلازما جاليسبتكم والميكوبلازما سينوفى. بالنسبة للإختبارات السيرولوجية أثبت كل من إختبارى تلازن الدم الإثباطى والإليزا أنهم أكثر دقة. وكانت أعلى نسبة إيجابية فى إختبار تلازن المصل السريع بإستخدام الأنتيجن الليبوزومى والأنتيجن التجارى المصبوغ. وأمكن فى هذا البحث الكشف عن الميكويلازما جاليسبتكم فى كيس المح لكتاكيت عمر يوم بطريقتى العزل البكتيريولوجى وسلسلة تفاعل البلمرة لتمييز الحمض النووى وثبت أن الإختبار الأخير أكثر دقة حيث أمكن تشخيص ٨٪ من الحالات بواسطته فى حين كانت سلبية بطريقة العزل البكتيريولوجى.

SUMMARY

Situation of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synovae (MS)* infections among different chicken flocks in Egypt showed that the infection rates were 100 % in commercial broiler flocks, 66% in layers and 40% in broiler parent flocks. Different serological tests, culturing and polymerase chain reaction (PCR) techniques were evaluated for diagnosis of MG and MS. Regarding serological techniques, Haemagglutination inhibition (HI) and ELISA tests proved more specific. The highest number of reactors

were detected by rapid plate agglutination test using stained commercial antigen or liposomal antigen. Detection of MG in yolk sac of 100 day old chicks by culturing techniques and PCR showed that no cases positive for culture were missed by PCR while 8% of cases were positive for PCR, but negative for culture.

Key words: *Chickens- Mycoplasma- Diagnosis- upper Egypt.*

INTRODUCTION

Mycoplasmosis in chickens continues to be a major problem in poultry industry specially in multiple age farms. *Mycoplasma gallisepticum* (MG) is responsible for chronic respiratory disease after complication with respiratory viruses, ND or IB live vaccines or E. coli. Economic losses are mainly due to. decreased egg production, reduced fertility and hatchability rates, poor growth, low feed conversion and increased culls.(Yoder, 1991)

Diagnosis of MG has been done by serological testing and culturing techniques. The rapid plate agglutination (RPA), haemaglutination inhibition (HI) and ELISA tests have been applied in serodaignosis of MG infection (Coleman,1982; Ansari et al. ,1983; Opitz et al.,1983; Piela et al., 1984; Czifra et al., 1995 and Shah-Majid, 1996). Problems with less-sensitive and non-specific reactions in serological tests have been reported (Kleven and Soliman, 1988 and Yoder, 1989). On the other hand, culture procedures may not always be successful, because the organism may be difficult to grow in artificial media. Sometimes, over growth of faster non-pathogenic Mycoplasmas can make culture procedures not successful. (Glisson et al., 1984).

A recent approach to improve diagnostic methodologies for pathogenic avian mycoplasmas is the development of DNA probe techniques (Razin, 1985; Khan et al., 1989 and Zhao and Yamamoto; 1990 a,b and 1993). Polymerase chain reaction (PCR) has been proved to be specific and sensitive method for diagnosis (Saiki et al, 1985; Mullis and Faloona, 1987 and Innis et al., 1990). Recent DNA probe-based kits are now available for MG from IDEXX - USA.

In Egypt, Mycoplasma infection was reported by many authors and was found to be the main cause of high economic losses in chicken flocks (El-shater, 1986 and Ibrahim et al.,1988). El-Shater (1986) recorded that the recovery rate of Mycoplasma was 18 % in 500 samples collected from 16 flocks of different ages at Cairo district.

Sokkar et al., (1986) isolated MG from chickens at a rate of 24 %. The isolates were found in association with *E. coli*, *Ps. Aeuroginosa*, *Staph. aureus* and *S. gallinarum pullorum*.

Soliman (1990) reported that serological screening of chickens of various ages and different localities in area of upper Egypt revealed that out of 200 serum samples, 138 samples were positive by using RPA and HI tests.

Laila et. al. (1993) recorded that out of 820 egg yolk samples, 172 (20.9 %) were Mycoplasma positive in RPA, 70 of them were MG and 102 were MS. Haemagglutination inhibition (HI) test revealed that 192 egg yolk samples (23.5 %) were positive, 81 and 111 possessed MG and MS antibodies respectively. On the other hand the recovery rate of Mycoplasma organism was 21.7 % (178 positive samples out of 820 egg yolk tested samples).

El-Dib (1994) reported that the isolation rates of Mycoplasma from the dead and sacrificed and tracheal swabs from living broiler birds were 8.09%, 13.1% and 11.7% respectively.

The purpose of the present study was:

1. To investigate the situation of MG and MS infections in broilers, layers and parent flocks in upper Egypt .
2. To evaluate the sensitivity of RPA, ELISA, culturing techniques and PCR reaction for MG diagnosis.

MATERIALS and METHODS

Rapid plate agglutination (RPA) antigen:

1. A liposomal antigen (Contains no serum additives) was provided by Dr. S. H. Kleven, Poultry Dis. Research Center, Athens, Georgia, USA. The test was carried out according to method described by Adler and Yamamoto, (1956).
2. MG and MS stained antigens were supplied by Intervet laboratory, Holland

Haemagglutinating antigen:

The antigen was kindly provided by Dr. S. H. Kleven, Poultry Dis. Research Center, Athens, Georgia, USA. The test was carried out after Yoder, (1980).

ELISA Kits:

The chicken MG and MS ELISA Kits (ProFLOK®) produced by Kirkegaard & Perry Laboratories Inc. (KPL), Maryland, USA were used in ELISA test.

Serum samples:

A total of 900 serum samples representing 45 chicken flocks (25 commercial broiler, 15 layers and 5 broiler parent flocks from El-menia, Assiut, Sohag and Kena provinces.) of different ages were collected and subjected for serological examination by using of RPA, HI and ELISA tests.

Culture media:

Brain-heart infusion (BHI) broth and agar (*Difco*) , were used for isolation of MG from yolk sacs of day old chicks (Yoder, 1980).

ELISA test:

The test was carried out according to Avakian et al. (1988). Coated plates were provided by Kirkegaard & Perry Lab. inc., Maryland, USA (KPL®). Sera were added to the coated plates for 30 minutes. Plates were washed three times, enzyme conjugate was added, then washed. Substrate was added, then stop solution. Absorpance at 405 nm was estimated using an automatic Biotek EL 311S reader. Titers were calculated with a software program of Profile.

RPA test:

The test was carried out according to the method described by Adler and Yamamoto (1956) in which one drop of RPA stained or liposomal antigen was mixed with one drop of the tested serum on a white glazed plate. The test was observed within two minutes as clumping of antigen in case of positive test.

HI test:

The test was carried out according to the method described by Yoder (1980).

Isolation and identification of MG:

100 day old chicks of broiler type were numbered and subjected to isolation of MG. Swabs from yolk sacs were taken for running PCR and simultaneously swabs were inoculated in BHI broth incubated at 37°C for 3-5 days and then cultured on BHI agar. Plates were incubated at 37°C for 3-5 days under reduced oxygen tension and then examined microscopically for presence of colonies, (Yoder, 1980). Mycoplasma isolates were identified by using growth inhibition test, (Clyde, 1964); growth precipitation test, (Krogsgarrd-Jensen, 1972) and immunofluorescent antibody technique, (Baas and Jasper, 1972).

Growth inhibition test:

Specific antiserum of MG was placed on media inoculated with unknown isolates and after appropriate incubation, the absence of growth in the presence of specific antiserum was recorded.

PCR for DNA detection:

The flock chek® PROBE of *Mycoplasma gallisepticum* DNA test kit was supplied by IDEXX-USA. Three stages were carried out for implementation of the MG DNA test kit from yolk sac swabs according to the directions of manufacturer:

1- Sample preparation:

a- Centrifugation at 16,000 xg.
b- Washing with sample wash solution and centrifugation 2 times.
c- Heating at 120° C to release DNA.

2- DNA amplification:

Place the tubes into DNA amplifier and activate PCR program. All DNA amplifications were performed in an automatic DNA thermocycler. The program was adopted as described by Nascimento et al. (1991).

3- DNA detection:

a- Dot 3 µl of each PCR amplificate onto the nylon membrane.
b- Set the membrane in 0.4 N NaOH.
c- add bloching solution.
d- Add conjugate and wash.
e- Add substrate.

Presence or absence of MG is based upon the presence of a coloured spot on the nylon membrane.

RESULTS

Results of RPA and HI and ELISA tests:

The results of serological screening for MG and MS by using RPA, HI and ELISA tests are shown in tables 1-6. Results indicated that RPA test was more sensitive than HI and ELISA tests in detection of antibodies against MG and MS. All tested 25 broiler flocks were infected with MG and MS as proved by serological tests

Results of PCR and culturing of MG:

The results of isolation of MG are summarized in table, 7. Results indicated that the use of PCR was more sensitive in detection of MG.

DISCUSSION

Serological tests as well as detection of Mycoplasma by culturing techniques have been effective traditional methods for diagnosis. All tested

25 broiler flocks were infected with MG and MS as proved by serological tests, 10 out of 15 layer flocks were infected with MG and MS. Broiler breeder flocks showed infection at a rate of 40 % as only 2 out of 5 flocks reacted positive.

Evaluation of the sensitivity and specificity of RPA, HI and ELISA as serological tests for monitoring antibodies to MG & MS were studied. Results showed that higher number of reactors (66 %, 54 % in broiler flocks, 40 %, 27 % in layer flocks and 35 %, 20 % in broiler breeder flocks for MG & MS respectively) were detected by RPA test in comparison with (50%, 42% in broiler flocks, 36 %, 21 % in layer flocks and 33 %, 16 % in broiler breeder flocks for MG & MS respectively) reactors were detected by HI test while (49%, 41% in broiler flocks, 34%, 20% in layer flocks and 30%, 15% in broiler breeder flocks for MG & MS respectively) were detected by ELISA test. Many authors have reviewed the more specificity of HI and ELISA tests than RPA test, (Mallinson, et al., 1981 and Avakian, et al., 1988). The less specificity of RPA may be attributed to false positive reactions due to the use of inactivated oil emulsion vaccines and variations in specificity of commercial antigens, (Glisson, et al., 1984 and Talkington et al., 1985). Liposmal antigen had higher degree of specificity in comparison commercial stained antigen. Similar results were reported by Avakian, et al. (1988).

For detection of the causative organism (MG) two techniques were carried out including culturing technique and polymerase chain reaction (PCR). Commercial kits produced by IDEXX, USA were used. In this technique DNA amplification procedure utilizes two synthetic DNA molecules whose properties are based on a DNA sequence specific to MG. These DNA molecules are used in conjunction with Ampli Teq DNA polymerase to amplify small amounts of MG target DNA. A third synthetic DNA molecule, labeled with enzyme is used as a sequence specific hybridization probe to provide colorimetric detection of the amplified DNA, (Khan et al., 1989; Kleven et al., 1988a, and Kleven et al., 1988b).

Our results proved that PCR probe-based kits are highly sensitive than culturing as no cases positive for culture were missed by PCR, while 8% of cases were positive for PCR, but negative for culture for MG . The same results have been recorded by Kleven (1993).

Because PCR is an extremely sensitive procedure care must be taken to prevent cross-contamination. Negative and positive control at proper concentration should be included in every PCR test to estimate the lower limit of specificity and upper limit of sensitivity.

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Table (1): Serological screening for MG in broiler flocks by using RPA , HI and ELISA tests

Flock No.	No. of Samples	RPA		HI test		ELISA test	
		Liposomal	Stained	No. of +ve	GM*	No. of +ve	Mean (n=20)
1	20	14	15	12	45	12	3240
2	20	13	16	11	44.8	10	2456
3	20	15	16	10	45	10	3652
4	20	12	14	9	43.1	9	2691
5	20	13	14	11	38.7	11	2540
6	20	14	16	10	45	10	3645
7	20	13	14	9	46	9	3598
8	20	14	15	11	42.8	11	3120
9	20	15	16	12	44.9	12	2987
10	20	12	15	10	43.1	10	2365
11	20	13	15	9	43	9	3120
12	20	10	14	8	45.1	8	3694
13	20	12	13	11	41.1	10	3012
14	20	14	15	10	31.2	10	2130
15	20	13	14	9	33.2	9	2143
16	20	16	17	11	34.2	9	2013
17	20	14	15	10	38.1	10	2654
18	20	11	13	9	38.2	9	2651
19	20	15	16	11	45.1	11	3546
20	20	14	15	10	45.6	10	3954
21	20	13	14	12	29.6	11	1965
22	20	12	13	8	30.5	8	2364
23	20	12	14	10	31.4	10	2431
24	20	15	15	11	32.1	10	2135
25	20	12	13	8	31.6	8	2154
Total	500	329	367	252		246	
%		66%	73%	50%		49%	

* GM= geometric mean titer

Table (2):Serological screening for MG in layers flocks by using RPA , HI and ELISA tests

Flock No.	No. of Samples	RPA		HI test		ELISA test	
		Liposomal	Stained	No. of +ve	GM*	No. of +ve	Mean (n=20)
1	20	13	14	12	29.4	11	1162
2	20	14	15	12	28.7	12	1430
3	20	12	15	11	39.4	11	1682
4	20	14	15	12	32.1	12	1954
5	20	13	15	12	35.4	11	1542
6	20	0	0	0	0	0	23
7	20	0	0	0	0	0	46
8	20	0	0	0	0	0	51
9	20	12	14	11	25.8	11	2954
10	20	13	14	12	35.4	10	1965
11	20	0	0	0	0	0	130
12	20	0	0	0	0	0	102
13	20	12	15	10	25.6	10	1658
14	20	9	10	8	24.9	8	1234
15	20	8	10	7	26.4	7	1126
Total	300	120	127	107		103	
%		40%	42%	36%		34%	

* GM= geometric mean titer

Table (3):Serological screening for MG in broiler parents flocks by using RPA , HI and ELISA tests

Flock No.	No. of Samples	RPA		HI test		ELISA test	
		Liposomal	Stained	No. of +ve	GM*	No. of +ve	Mean (n=20)
1	20	0	0	0	0	0	41
2	20	0	0	0	0	0	39
3	20	18	20	17	46	15	3951
4	20	0	0	0	0	0	21
5	20	17	20	16	45.4	15	3624
Total	100	35	40	33		30	

* GM= geometric mean titer

Table (4): Serological screening for MS in broiler flocks by using RPA , HI and ELISA tests

Flock No.	No. of Samples	RPA		HI test		ELISA test	
		Liposomal	Stained	No. of +ve	GM*	No. of +ve	Mean (n=20)
1	20	9	10	8	35	8	2693
2	20	8	9	7	36.1	7	2876
3	20	10	11	7	29.5	7	1954
4	20	11	12	10	36.5	9	2965
5	20	12	13	9	37.1	9	2164
6	20	14	15	11	45	10	3982
7	20	12	15	10	46	10	3549
8	20	10	11	8	32.5	8	2116
9	20	11	12	7	44.9	7	2987
10	20	10	13	9	39.8	9	2114
11	20	13	14	8	38.6	8	3120
12	20	14	16	10	45.1	9	3798
13	20	10	13	9	41.1	9	3621
14	20	9	10	6	29.8	6	1945
15	20	10	12	8	33.2	8	2004
16	20	11	12	9	30.5	9	2310
17	20	10	12	7	38.1	7	2654
18	20	11	12	9	38.2	9	2365
19	20	8	9	7	20.6	7	1546
20	20	10	12	8	39.2	8	1254
21	20	10	12	7	40.1	7	1987
22	20	11	13	6	41.1	6	1632
23	20	11	13	10	41.1	10	1985
24	20	12	14	10	40.3	8	2487
25	20	14	15	9	40.9	9	2365
Total	500	271	308	208		203	
%		54%	62%	42%		41%	

* GM= geometric mean titer

Table (5):Serological screening for MS in layers flocks by using RPA , HI and ELISA tests

Flock No.	No. of Samples	RPA		HI test		ELISA test	
		Liposomal	Stained	No. of +ve	GM*	No. of +ve	Mean (n=20)
1	20	9	10	8	12.9	7	986
2	20	8	10	7	11.2	7	1364
3	20	9	10	6	9.4	6	965
4	20	8	10	7	8.6	7	1143
5	20	12	13	7	12.6	7	978
6	20	0	0	0	0	0	67
7	20	0	0	0	0	0	103
8	20	0	0	0	0	0	65
9	20	8	10	7	18.4	6	1164
10	20	9	11	7	11.6	7	1583
11	20	0	0	0	0	0	96
12	20	0	0	0	0	0	68
13	20	5	8	4	6.8	4	1123
14	20	6	8	5	5.4	4	1023
15	20	7	9	5	6.8	5	896
Total	300	81	99	63		60	
%		27%	33%	21%		20%	

* GM= geometric mean titer

Table (6):Serological screening for MS in broiler parents flocks by using RPA , HI and ELISA tests

Flock No.	No. of Samples	RPA		HI test		ELISA test	
		Liposomal	Stained	No. of +ve	GM*	No. of +ve	Mean (n=20)
1	20	0	0	0	0	0	56
2	20	0	0	0	0	0	100
3	20	11	12	8	26	7	2530
4	20	0	0	0	0	0	21
5	20	9	11	8	16.6	8	1265
Total	100	20	23	16		15	
%		20%	23%	16%		15%	

* GM= geometric mean titer

Table (7): Detection of MG in yolk sac of day-old-chicks By isolation and PCR

		Culture	
		+	−
PCR	+	16	8
	−	0	76