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EVALUATION OF MULTIPLEX PCR BASED TEST IN COMPARISON WITH CULTURE METHOD FOR DETECTION OF *MYCOPLASMOSIS* IN INFECTED CHICKENS

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SUMMARY

A total of 94 chicken samples were examined for *Mycoplasma gallisepticum* and *Mycoplasma synoviae* either by culturing procedure or by using the multiplex PCR. The comparative analysis of the obtained results indicated that the multiplex PCR was more sensitive and reliable in the detection of both organisms, where the multiplex PCR detected *Mycoplasma gallisepticum* in 20.21% of the tested samples while it gave 5.319% prevalence for *Mycoplasma synoviae*. Moreover isolation and culturing procedures detected *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in a prevalence rate of 15.956% and 2.127% of the tested samples comparatively. So these results concluded that, the multiplex PCR has become a valuable and easy test aiding in diagnosis of *M. gallisepticum* and *M. synoviae* infection in chickens.

INTRODUCTION

Mycoplasmas belongs to the class *Mollicutes* and are among the smallest free living Micro-organisms capable of auto-replication. Mycoplasmas specially *M. gallisepticum* is a major problem in poultry industry worldwide causing chronic respiratory diseases of chickens and turkeys. This disease causes substantial economic losses due to decreased egg production and hatchability and condemnation of the infected flocks. *Mycoplasma gallisepticum* is commonly involved in chronic respiratory diseases in chickens and infectious sinusitis in turkeys and infection are often complicated by *E.coli* and / or respiratory viruses ***(Stipkovits andKempf 1996)***. *Mycoplasma synoviae* may causes sub-clinical or clinical infections. Some strains cause air saculitis on their own or in mixed infection with other bacteria or respiratory viruses. Exudative synovitis of joints and tendon sheathes may occur as a result of systemic infection with *Mycoplasma synoviae* ***(Kleven 1997)***. Although

detection of Mycoplasma is still carried out routinely by serological and cultural methods; efforts have been made in recent years to establish polymerase chain reaction (PCR) based tests which allow more rapid diagnosis than culture but have comparable sensitivity and specificity ***(Kempf 1998)***. So PCR has become a valuable supplemental test to aid in the diagnosis of Mycoplasma infection. The primary advantage of PCR is that it is a rapid and sensitive method for direct detection of the organism's nucleic acid in tracheal swabs. So this study aimed to compare multiplex PCR based test and culture method for detection and diagnosis of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in infected chicken.

MATERIALS AND METHOD

1-Standard Strains and Antisera.

Either *M. gallisepticum* (S6) strain and *M. synoviae* (local isolates) and their antisera were supplied by the central lab for evaluation of veterinary biologics (CLEVB), Abbasia, Cairo.

2-Samples for isolation of Mycoplasma.

A total of 94 Samples including 15 tracheal swabs, 14 nasal sinus swabs, 21 trachea, 15 lung tissues, 22 air sacs and 7 synovial fluid were collected from chickens at different ages.

3-Primary inoculation.

All obtained samples were transferred into 10 ml of Frey's medium and then divided equally into two parts, the first part was used for culturing and isolation procedures and the second one was used for polymerase chain reaction.

4-Isolation of *Mycoplasmas*.

The procedure was attempted as described by ***Ley et al (1997)***. The primary inoculation broths were incubated at 37°C with humidity till the colour of media changed from pink to orange yellow. A week later, the unchanged broth cultures were passaged to a new broth media. Samples which changed the colour of media were streaked onto Frey's agar then incubated at 37°C with humidity and 5 % CO_2 and checked for colonies using stereoscopic microscope for at least two weeks. The suspected colonies were subjected to digitonin sensitivity, tetra-zalium, reduction, and glucose fermentation tests and also to growth inhibition test using specific *M. gallisepticum* and *M. synoviae* antisera.

5-DNA isolation.

After incubation and colour changing of the primary inoculation, the broth extracted for the DNA template as described by ***Lauerman (1998)*** by centrifugation at 15000 xg, washing with distilled water, followed by boiling for 10 min, and then kept at -20°C for 10 min,

centrifugation and collecting the supernatant at -20°C until use.

6-Multiplex PCR.

(A) Primers.

The *M. gallisepticum* primers consists of the following sequences ... (MGF: 5`-GAG CTA ATC TGT AAA GTT GGT C-3`) and MGR: 5`-GCT TCC TTG CGG TTA GCA AC-3`) ***(OIE 2008)***, while the *M. Synoviae* primers sequences were (MSF: 5`-GAA GCA AAT AGT GAT ATC A-3`) and (MSR:5`-GTC GTC TCG AAG TTA ACA A-3`) ***(Pang et al. 2002)***

(B) PCR.

The reaction mixture was prepared for one 50μ PCR reaction as follow (34.75 μ ultra pure H2o, 5μ10x buffer, 1μ dNTP (10 mM), 0.5 μ of each primer (20 pmole/μ), 0.025μTaq DNA polymerase (5μ/ml), 2μ Mgcl2 (50mM) and 5μ of DNA templates. The tubes were placed in a thermal cycler for the following programs; 40 cycles: 94°C/30 sec, 55°C/ 30 sec., 72°C/60 sec.), cycle final extension 72°C/5min and soak at 4°C. PCR products were detected by funning in 2% agarose gel electrophoresis incorporating 100bp marker followed by examination under U.V light.

RESULTS AND DISCUSSION

It has been seeking for sometime a simple, accurate and reproducible procedure for isolation and typing of organism. The primary objective of the present study aimed to reach this target and to differentiate between culturing and PCR procedures to develop the simultaneous detection and differentiation of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in chickens. With the multiplex PCR detection and differentiation of both Mycoplasmas could be achieved in a single reaction which greatly improves the rapid detection of these pathogens ***(Ben Abdelmoumen et al., 2005)***.

Table. (1) Detection of *M. gallisepticum* and *M. synoviae* by culturing and isolation procedure

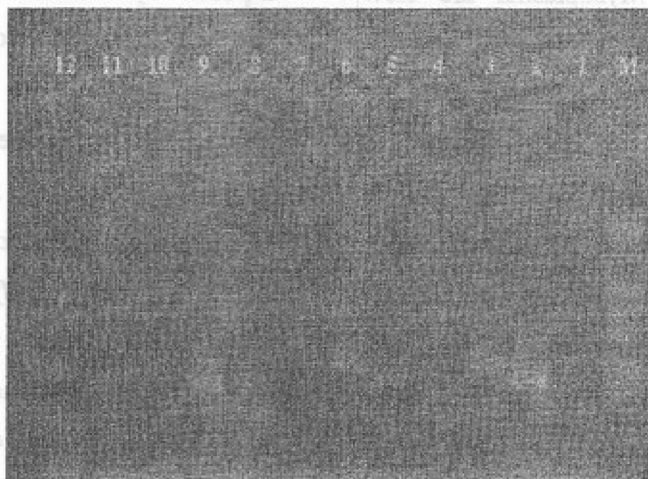
Samples		*M. gallisepticum*		*M. synoviae*		Grand Total	
Type	Number examined	No. of positive isolates	Recovery rate	No. of positive isolates	Recovery rate	No. of positive isolates	Recovery rate
Tracheal swab	15	3	20.0%	0.0	0.0%	3	20.00%
Nasal swab	14	0.0	0.0%	0.0	0.0%	0.0	0.0%
Trachea	21	5	23.80%	0.0	0.0%	5	23.80%
Lung tissue	15	2	13.33%	0.0	0.0%	2	13.33%
Air Sacs	22	5	22.72%	1	4.55%	6	27.27%
Synovial fluid	7	0.0	0.0%	1	14.28%	1	14.28%
Total	94	15	15.956%	2	2.127%	17	18.085%

Regarding culturing and isolation procedures, the present results as shown in table (1) revealed that 15 out of 94 samples were positive to *M .gallisepticum* from either tracheal swab (3 out of 15) , trachea (5 out 21) , lung tissue (2 out of 15) and air sacs (5 out of 22) and 2 out of 94 samples were identified as *Mycoplasma synoviae* from either air sacs (1 out of 22) and synovial fluid (1 out 7), in a prevalence rate of 15.956% and 2.127% for both *M .gallisepticum* and *Mycoplasma synoviae* respectively, while the total number of recovery was 17 isolates out of 94 examined samples giving rise a recovery rate of 18.085% . These findings come parallel to those of ***Dardeer (1996)*** who investigated Mycoplasma organisms from the respiratory organs of the affected chicken and the highest recovery rate was from trachea (9.6%) followed by air sacs (5.5%) and lung tissue (4.2%) while the total recovery rate was 19.2% for *M .gallisepticum.* Also ***Yamamoto et al (1992)*** isolated Mycoplasma from most of oropharyngeal swabs and mentioned that the isolation rate was higher in layer than broiler chicken. More over ***Shaker (1995)*** stated that the isolation rate obtained from trachea was 8.5%, air sac was 8.0% and lung was 1.3%.

Table. (1) Detection of *M. gallisepticum* and *M. synoviae* by multiplex PCR .

Samples		*M. gallisepticum*		*M. synoviae*		Grand Total	
Type	Number examined	No. of positive isolates	Recovery rate	No. of positive isolates	Recovery rate	No. of positive isolates	Recovery rate
Tracheal swab	15	3	20.0 %	1	6.66%	4	26.66%
Nasal swab	14	1	7.14%	0.0	0.0%	1	7.14%
Trachea	21	6	28.57%	1	4.76%	7	33.33%
Lung tissue	15	2	13.33%	0.0	0.0%	2	13.33%
Air Sacs	22	7	31.81%	1	4.545%	8	36.367%
Synovial fluid	7	0.0	0.0%	2	28.57%	2	28.57%
Total	94	19	20.21%	5	5.319%	24	25.53%

Fig.(1) agarose gel electrophoresis of multiplex PCR amplified products.



(Lane M: 100 bp Marker, Lane 1: Negative control, Lane 2:MG Positive control, Lane 3:MS Positive control, Lane 4,7,10,11&12 Negative PCR samples, Lane 5,8&9 MG Positive PCR products and Lane 6: MS Positive PCR product.).

of 15 from lung tissues and 7 out of 22 from air sacs samples. Giving rise *M .gallisepticum* prevalence rate of 20.21%. As regards to *Mycoplasma synoviae* the prevalence detection rate was 5.319% where 5 samples out of 94 were positive giving 207 bp molecular weight

Concerning the detection rate obtained by multiplex PCR, the results shown in (Table 2) and Fig. (1) revealed that 19 out of 94 samples were positive for *M .gallisepticum* giving a molecular weight of PCR product at 185 bp. These positive samples were obtained as 3 out

mycoplasmosis showed that 12 out of 15 samples produced a specific band. On comparison of culturing and isolation method with the multiplex PCR in the detection of *M. gallisepticum* and *M. synoviae* in the present results , it is clear that, the multiplex PCR was more sensitive and faster than the traditional isolation procedure, where it gave a total prevalence detection rates of 25.53% compared with 18.085% in culturing and isolation procedures. So it could be concluded that, Culture represents the performance standards for direct detection, but Mycoplasma are slow growing, relatively fastidious that require one or more weeks for growth and identification. In some cases isolates of Mycoplasma is unsuccessful as a result of the culture overgrowth of saprophytic Mycoplasma that inhabit in the upper respiratory tract particularly in older chicken. The primary advantage of PCR is that, it is rapid and sensitive method of direct detection of the organism's nucleic acid compared with isolation techniques which are time consuming and may be problematic. multiplex PCR has become a valuable , rapid and easy test aiding in diagnosis of *M. gallisepticum* and *M. synoviae* infection in chickens.

product with the multiplex PCR and these samples were 1 out of 15 tracheal swabs, 1 out of 21 from trachea, 1 out of 22 from air sacs and 2 out of 7 from synovial fluid samples. These results indicated that the multiplex PCR was able to detect and differentiate the presence of either *M. gallisepticum* and or *M. synoviae* in infected chickens. In this respect ***Pang et al (2002)*** demonstrated that the multiplex PCR assay was specific and sensitive to detect and differentiate the presence of the respiratory pathogens in infected chicken as well as chicken exposed by lateral transmission. ***Saad and Dirgham (2008)*** in Jordan screened 76 different chicken flocks with respiratory symptoms serologically by commercial MG-ELISA kit and bacterial isolation for Mycoplasma. 24 *Mycoplasma gallisepticum* isolates were cloned and the presence of *Mycoplasma gallisepticum* in the cloned cultures were confirmed by PCR and the prevalence of *Mycoplasma gallisepticum* by isolation was 31.6%. Moreover ***Ghaleh et al., (2005)*** reported that the sensitivity of PCR method for detecting Mycoplasma species was equivalent to about 100 CFU/PCR traction mixture. Also mentioned that PCR analysis of non cultivated samples collected from chicken suspected to have

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تقيم إختبار تفاعل البلمرة المتسلسل بالمقارنة بطريقة العزل التقليدية لتشخيص العدوي بالميكوبلازما في الدجاج

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الملخص العربي

تم فحص عدد ٩٤ عينة من الدجاج لميكروب الميكوبلازما جاليسبتكم والميكوبلازما سينوفي بإستخدام طريقة الزرع و العزل التقليدي وكذلك بإستخدام تفاعل البلمرة المتسلسل المتعدد وبمقارنة نتائج الاختبارين تبين أن تفاعل البلمرة المتسلسل اكثر حساسية في الكشف عن الميكروبين في العينات التي تم فحصها حيث أن تفاعل البلمرة المتسلسل كشف عن الميكوبلازما جاليسبتكم في ٢٠,٢١% وعن الميكوبلازما سينوفي ٥,٣١٩% بينما إختبار العزل التقليدي كشف عن الميكوبلازما جاليسبتكم في ١٥,٩٥٦% وعن الميكوبلازما سينوفي ٢,١٢٧% من العينات المختبرة . كما أن تفاعل البلمرة المتسلسل المتعدد يوفر الوقت ويعطى إمكانية فحص الميكروبين فى زات العينة فى نفس الوقت .