Differentiation of Mycoplasma synoviae strains in IRAN by PCR

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Abstract: Mycoplasma synoviae is one of the most important economic challenges in keeping the chickens all over the world and particularly in Iran which leads to chronic respiratory disease in chickens and turkeys. Difference in mycoplasma synoviae methods particularly in the countries that Livestock and Poultry are vaccinated with a live vaccine is a matter of great magnitude. In this paper, 1600 serum samples of 80 broiler breeder flocks were sampled on which RSA test was carried out in the Serology Laboratory. Then sampling for PCR and DNA extraction was performed through proposed methods. The results of RSA test revealed that from among 1600 serum sample taken from 80 farms, 68 farms became positive in RSA test. In General, from 1600 obtained samples, 1327 samples were positive in RSA test, 216 samples were negative, and from 57 suspected serum samples, 31 samples were reported as positive in the one-eighth dilution. Comparison of bands, obtained from the primers VlhA-F and VlhAR2, indicates that there is a difference between different strains of Iran and the vaccine strains MS-H and standard strain in terms of size. In PCR test with external primers, MSCons -F and MSCons-R, the band 350-400 indicates presence of Mycoplasma synoviae and MS-H. In general, using the DNA-diagnosis-based methods like PCR and PCR_RLFP in order to detect the Mycoplasma synoviae directly from the laboratory tissue or strains seems necessary. These methods are able to provide the detailed results in the presence of infections mixed with several Mycoplasmas, facing with secondary bacterial infections, Mycoplasma growth inhibitor such as antibiotics, or other host factors within one to two days

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1- Introduction

Mycoplasmas are from the Mollicutes class, have DNA, and need the Cholesterol and optimum temperature equal to 37oC for their growth (Fiorentin et al, 2013). So far, 23 different Mycoplasma strains have been taken from the birds and the host of their 16 strains is related to the poultries and turkey (Bradbury, 2005). In this regard, only four strains are pathogenic for the birds such as Mycoplasma gallisepticum, Mycoplasma synoviae, Mycoplasma meleagridis, and Mycoplasma iowae (Jordan, 2002). However, Mycoplasmas are existed in the poultry flocks and the Mycoplasma infection is still a major problem of poultry industry (Noormohammadi, et.al, 2007) and in the case of infected valuable breeder flocks, they may be killed or thus lose their value of export and sales (Saif et al, 2008). Therefore, because of the important pathogenic role of poultry Mycoplasmas, the eradication program from the breeder flocks has been implemented since the past years, but the detection of Mycoplasma type is the biggest problem in this regard (Hammond et.al, 2009). So far, numerous efforts have been made in order to identify and differentiate the Mycoplasma synoviae strains (Hammond et al, 2009). However, the identification of these strains has been less done in Iran and the characteristics of existing strains are not completely clear yet. Therefore, this research is seeking to differentiate and report the Mycoplasma synoviae strains in Iran.

Mycoplasmas genomes contain a significant percentage of repeated sequences. For instance, the sequence MaPa in Mycoplasma genitalium accounts for approximately 4.7 percent (Nathan and Gasser, 2007). Moreover, a large number of sequences and the biggest gene VLhA in Mycoplasma gallisepticum account for about 10.4 percent of the whole genome (Hong, 2004). These sequences play their roles in the anti-genetic diversity which is created on the cell (Bradbury, 2005). Discovering the anti-genetic size and phase diversity has been probably as one of the biggest advances in the Mycoplasma research. However, this size diversity can also play a key role in the host defense against the Mycoplasma (Noormohammadi et al, 2000).

Numerous studies have been conducted in the field of detecting Mycoplasma synoviae, and various methods have also been provided such as HI, SPA, ELISA, and PCR. Lauerman et al (1993) designed

Mycoplasma species-specific primers, named MS-1 and MS-2, according to the sequence 16S rRNA. They have indicated that by applying this primer the PCR test will have the sensitivity 81% and the specificity equal to 100% compared with other methods. Silveira et al (1996) examined the mentioned methods and determined the method PCR[1] as the fastest method. Furthermore, they indicated that the method of using the phenol for extracting DNA is the same as the Non-phenolic method. Therefore, without-phenol PCR method has also been applied in this research. Hong et al (2004) considered the end of N Terminal, the VlhA gene, which encodes the hemagglutinin, as the Mycoplasma synoviae alternative part and used it as the detection of Mycoplasma synoviae field sample in commercial poultries. Mardassi et al (2005) applied the primers MS1.2F0 MS1.2R0 and MS1-2Rli MS-2Fli for detecting Mycoplasma synoviae through the Duplex PCR with an emphasis on the gene VlhA. The results of their research indicate that this method is sensitive and specific enough to be used for differentiating Mycoplasma synoviae from Mycoplasma gallisepticum in co-infection. Nathan and Gasser (2007) could put these strains into 10 profiles from A to J through the specific primers of gene VlhA as well as applying SSCP test and HRM measurement method in 35 different strains of Mycoplasma synoviae. In this study, the specific primers of MScons-F and MScons-R with 98% sensitivity are used in order to detect the Mycoplasma synoviae strains. This is 6% higher than the sensitivity of PCR.

3- Materials and Methods

In this study, 1600 serum samples of 80 broiler breeder flocks were sampled. In order to perform RSA test in the Serology Laboratory, a volume of serum equal to 30 microliters is spilled on a white tile and a volume of color Mycoplasma synoviae antigen is added to it. The tile is shaken rotationally in order to mix the antigen and serum. In the presence of antibody against Mycoplasma synoviae, the agglutination phenomenon occurs in maximum 2 minutes and it is determined when the suspended particles in the fluid come together. According to the suggestion by Bradbury (2005), all positive serums at the previous stage were put in the in the Water bath 56 °C for 30 minutes in order to confirm the diagnosis of Mycoplasma synoviae and inactivating the non-specific reactions. In this study, the strains, contained in the Table (5), are studied in order to investigate the existence of different Mycoplasma types. These strains were previously studied by Ghorashi et al (2010) and have been studied in this research for the first time in Iran. The melting point and gen bank are provided in this table.

The suggested method in four studies (Bradbury, 2005) (Jordan, 2002) (Noormohammadi et al, 2007) (Hong et al, 2004) was used for sampling the PCR and DNA extraction, so that the sterile swabs were prepared from the palatine cleft, trachea, lungs, and air sac. Three swabs from three birds were put into a test tube containing 1 ml of PBS and transported to the laboratory in order to be used for performing the PCR. For DNA extraction by the Phenol-chloroform method, first 500 microliters of sample was centrifuged at 13000 rpm for 2 minutes after putting in the micro-tube. Samples, containing the larger particles, were first centrifuged at 3000 rpm for 5 minutes and then the supernatant was transferred to a new tube and the tube containing the precipitated materials was removed. After the centrifugation, the sample tubes were evacuated to 100 microliters. Then the lysis buffer with the same volume as the remainder with the quality of components was added to the sample tube according to the Table 1. The sample tube was put in the water bath 56 °C for 4 hours after shaking in the mixer for 2 minutes. In the lysis Buffer solution, tris with the PH 8 acts as the buffer and EDTA acts as the inhibitors of DNAasc enzymes. Furthermore, SDS compound is applied for dissolving the lipids in the cell membrane, and Proteinase K is used for protein digestion and intercellular junctions.

At this stage, the sample tube contains DNA which is obtained from the proteins and lipids; however, a mixture of extra materials, called crude lysate, is found in the composition of sample tube and it should be extracted. After the water bath, the saturated phenol with the same volume as the sample tube content was added to the samples in order to separate DNA from the crude lysate. After adding the phenol, the sample tubes were well shaken in the bench-top centrifuge at 13,000 rpm and room temperature for 15 minutes. At this stage, the sample tubes became the two-phased and supernatant phase was extracted by using a sampler and transferred to the new tube along with the mixture 50% with the equal volume of phenol-chloroform. Finally, 3M sodium acetate was added to 10% of tube volume and gently mixed in order to concentrate and deposit the extra DNA and 100 to 96 degrees cold absolute alcohol was added equal to two times higher than the sample size. The samples were put in the freezer at -20 °C for 15-20 minutes. The samples were then brought out of the freezer and were centrifuged for 15 min at 13000 rpm. The tube supernatant was evacuated and placed under the hood after shaking in order to dry the alcohol in the tube. 50 Microliters of sterile distilled water was added to the sample in order to avoid the excessive drying and breaking DNA.

[1] polymerase chain reaction

According to the proposed method by Terrestrial Manual, the Polymerase chain reaction (PCR) method is used in this study in order to confirm the samples (OEI, 2008). Applied primers are specified for the gene VlhA -PCR in Mycoplasma synoviae (Lauerman et al, 1993). These primers have been applied in order to identify the MS in pure culture or the clinical sample (Nathan and Gasser, 2007). Primers and their nucleotide sequences are shown in Table (2). Moreover, all reactions were done in the thermal cycler Gradient Mastercycler, made in Eppendroff Germany, according to the Time and thermal schedule as shown in the Table (3). Finally, the reaction PCR was done in the volume 25 Microliters and applied in all positive and negative simultaneous control cases with similar composition of Table (4).

Electrophoresis was made according to Hoseini's suggestion (2002). In this plan, the comb, glass plates, and adjacent blades were fully cleaned through a detergent and rinsed with deionized distilled water. The glass plates were fully dehydrated by using the ethanol 95% and dried in air. Gel mold was closed by placing the adjacent blade on the inner surface of back plate and then putting the front plate on the blades. So that the blades were put a little more outer than the edge of glass plates, thus it was gently pressed after placing the front plate glass of blades in order to be put in its place between two plates. Around and below the mold was sealed completely with a high-quality tape. Furthermore, the applied agarose gel in this study was agarose 2% which was weighed according to the target percentage of agarose powder, and the buffer TBE, which was a mixture of Tris base, boric acid and EDTA, was added to it. Gel solution was poured into the mold carefully and gently with no air bubbles. For the purpose, the air bubble remover was used. Level meter was used in order to spread the gel uniformly at all levels of electrophoresis tank. Then it was put into the gel and in its place, and the gel was put at room temperature until the completed solidification. Then the comb brought out and the gel was placed in the electrophoresis system and the solution of 2% TBE was poured into the tank until the surface of gel was completely covered. 10 microliters of PCR product was mixed with Two microliters of loading buffer and put in the gel holes. After finishing the sample loading, the electrodes were connected to the power supply and the gel was put at a constant current of 100 V for 45 minutes. The gel was placed on the UV system (BioRAD, Bio-USA, California, Rad lab) and a photo was taken of it. Furthermore, the quite saturated fluorescent colors were used in the central laboratory of Khorasan Razavi Veterinary Organization for PCR - HRM HIGH RESOLUTION MELTING Analysis which acts on the basis of determining the melting point of PCR product.

4- Results

The results of RSA test indicated that from 1600 serum samples, taken from 80 farms, 68 farms became positive in RSA test. In General, from 1600 obtained samples, 1327 samples were positive in RSA test, 216 samples were negative, and from 57 suspected serum samples, 31 samples were reported as positive in the one-eighth dilution. These samples showed the band 350-400 on the agarose gel in PCR test with external primers, MSCons -F and MSCons-R, as shown in Figure (1); this indicates the presence of Mycoplasma synoviae and MS-H. Moreover, according to the Figure (1), the comparison of bands, obtained from the primers VlhA-F And VlhAR2, indicates that there is a difference between different strains of Iran and the vaccine strains MS-H and standard strain in terms of size, so that the band, related to the vaccine strain, is significantly different with the band related to the standard strain WVU1853 as well as other strains. However, there is no difference between different samples in terms of band size by using the primers related to the gene 16srRNA. These findings are consistent with the results of research by Bencina et al (2001), Hammond et al (2009) and also Jeffery et al (2007). Therefore, the initial differentiation of strains and vaccine strains can be possible (provided the product size difference) by using the primer related to the gene VlhA; Furthermore, this PCR product is more valuable in the complementary molecular studies such as RFLP sequencing compared with the gene 16srRNA because of covering a section of VlhA polymorphic area (Hong, et.al, 2004).

5- Discussion and conclusion

Given the importance of Mycoplasma synoviae disease in terms of economic aspect and according to the emergence of respiratory and arthritic disease and with regard to the doubled costs of health care and lack of proper response and lack of maximum performance of the birds infected with this bacteria, the DNA-diagnosis-based methods should be used in order to detect the Mycoplasma synoviae directly from the laboratory tissue or strains (Ramirez, 2006). Other methods have been created for simultaneous diagnosis of Mycoplasma types in clinical samples such as the Multiple PCR and PCR_RLFP. Results of PCR are determined within one to two days, while it takes one to three weeks for the cultivation and identification of organism. Furthermore, PCR is able to provide the detailed results in the presence of infections mixed with several Mycoplasmas, facing with secondary bacterial infections, Mycoplasma growth inhibitor such as antibiotics, or other host factors (Noormohammadi et al, 2007). Particularly, the saprophytic Mycoplasmas, which have the faster growth in the enriched culture than the Mycoplasmas synoviae, are among the most important problems of culture.

6- Appendix

Table 1: Composition of the component percentage of lysis Buffer

Component	Amount	Unit
Tris-Hcl	50 (PH = 8)	$mLit$
SDS	1 %	----
NaCl	100	$mLit$
EDTA	50	$mLit$
Proteinase K	2	$\frac{mgr}{\mu l}$

Table 2: Details of primers and specific nucleotide sequences of gene VlhA -PCR in studied Mycoplasma synoviae

Primers	Nucleotide sequence	Source
MScons-F	TACTATTZGCAGCTAGTC	Nathan and Gasser, 2007
MScons-R	AGTAACCGATCCGCTTAAT	Nathan and Gasser, 2007
VlhAF	ATTAGCAGCTAGTGCAGTGGCC	Benčina et al. 2001
VlhAR1	CAGCGCTAGTTTTTGTTTTTTGG	Benčina et al. 2001
VlhAR2	AGTAACCGATCCGCTTAATGC	Hammond, et.al 2009

Table 3: Time, thermal and number of rotations

Stage	Temperature (^{o}C)	Time (Min)	Number of rotations (rpm)
Initial denaturation	93	2	1
Secondary denaturation	95	15	35
Primer binding	52	15	35
Initial expansion	70	20	35
Secondary expansion	70	5	1

Table (4): The combination of simultaneous positive and negative control in the PCR reaction

Component	Mount	Unit
F primer ($20\left[\frac{pmol}{\mu Lit}\right]$)	0.15	$[\mu Lit]$
R primer ($20\left[\frac{pmol}{\mu Lit}\right]$)	0.15	$[\mu Lit]$
dNTP (10 mM)	0.75	$[\mu Lit]$
$MgCl_2$ (50 mM)	2.00	$[\mu Lit]$
PCR Buffer	2.5	$[\mu Lit]$
Water	17.4	$[\mu Lit]$
Taq DNA polymerase ($\frac{5U}{\mu Lit}$)	0.10	$[\mu Lit]$
Template DNA	1.94	$[\mu Lit]$

Table 5: Mean±SD of the melting points and GCP for each strain following PCR analysis

Genotype (number of times tested)	Number of isolates/batches tested	Peak 1 (6C)	Peak 2 (6C)	Peak 3 (6C)	GCP±SD
ts-11 (125)	3	76.5±0.3			96.1±3.6
K1659 (30)	1	75.2±0.1	76.9±0.1		97.9±2.5
K1453 (28)	1	77.7±0.1			98.1±1.5
87006 (21)	1	74.9±0.1	79.1±0.1		98.9±0.3
87081 (18)	1	74.8±0.9	76.4±0.7	77.6±0.4	95.0±2.1
F (58)	1	78.2±0.1			98.4±1.6
S6 (28)	1	78.2±0.1			99.2±0.7
6/85 (38)	1	74.8±0.1			96.9±2.2
86134 (42)	1	75.3±0.8	77.3±0.3		98.4±1.4
Ap3AS (21)	1	75.7±0.1	77.1±0.4		98.2±1.2

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