JWPR
Journal of World's Poultry Research

2018, Scienceline Publication
J. World Poult. Res. 8(3): 50-58, Sep 25, 2018
Research Paper, PII: S2322455X1800008-8

Real Time PCR Quantification and Differentiation of both Challenge and Vaccinal *Mycoplasma gallisepticum*s trains Used in Vaccine Quality Control

Rafik Hamed Sayed[1], Hanan Ali Ahmed[1], Fady Abdelmohsen Shasha[1] and Abdelhakim Mohmoud Ali[1]

Central Laboratory for Evaluation for Veterinary Biologics (CLEVB), Abbasia, Cairo, Egypt

*Corresponding author's email: rafikhamed1010@hotmail.com

Received: 30 July 2018
Accepted: 01 Sept 2018

ABSTRACT

Mycoplasma gallisepticum is an economically important pathogen of poultry worldwide, causing chronic respiratory disease in chickens and turkeys. Vaccination of poultry with *Mycoplasma gallisepticum* live vaccines is an approach to reduce susceptibility to infection and to prevent economic losses. The goal of this study was to develop an alternative method for evaluation of live and killed vaccine using quantitative differential real time PCR (rt-PCR) assay. Real time PCR assay was implemented for titration and identification of three types of *Mycoplasma gallisepticum* (F, ts-11 and field strain). Three groups of chicks were vaccinated by using F- strain, ts-11 and killed vaccine and the forth group was considered control. Challenge test was appliedby using *Mycoplasma gallisepticum* field strain (10^8 CFU) at three weeks post vaccination. Antibody ELISA titers against *Mycoplasma gallisepticum* were 319, 259 and 1009 for F, t-11 and killed vaccine respectively at 3 weeks post vaccination. The protection rates were 81.5%, 74%, and 66.6% for F- strain, ts-11 and killed vaccine respectively that was determined by air sac lesion scour. Using quantitative differential rt-PCR for necropsied birds at 5 days post challenge 7days post challenge and 14 days post challenge demonstrated that the F-strain vaccine had ability to prevent shedding of field strain at 14 days post challenge mean while the ts-11 and killed vaccine decreased shedding of field strain from $10^{8.1}$ and $10^{8.6}$ to $10^{5.1}$ and $10^{5.8}$CFU respectively at 14 days post challenge. In this study, rt-PCR had ability to identify and quantify of two types of vaccines (F and ts-11) and field strain.

Keywords: *Mycoplasma*, rt-PCR, Vaccine, Poultry

INTRODUCTION

Mycoplasma gallisepticum (MG) infects a wide variety of gallinaceous birds including chickens, turkeys and pheasants (Yoder,1990). MG is the most important notifiable disease. MG is a cause of chronic respiratory disease, especially in the presence of other respiratory microorganism or environmental stresses. The disease is characterized by coryza, conjunctivitis, sneezing, and sinusitis particularly in turkey and game birds. It results in loss of production, downgrading of meat-type birds and loss of egg production. MG strains vary in infectivity and virulence, and infections may sometimes be inapparent (OIE, 2013). Vaccination with bacterin has been shown to control, but not eliminate colonization, it is felt that bacterins are of minimal value in long-term prevention on commercial layer (Ley, 2008; Moraes et al., 2013). Live vaccines that have been used to control MG include F strain (Burnham et al., 2002), and more recently, ts-11 (Whithear et al., 1990). Culture methods, are often labor-intensive and require specially formulated media, so the improvement of diagnostic tools for direct detection of mycoplasma is necessary (Feberwee et al., 2005).

The quality control of live MG vaccine depends on identity, titration, safety, sterility and potency tests. The identity test was determined by conventional Polymerase Chain Reaction (PCR) that does not differentiate between the types of live MG vaccines (Mettifogo et al., 2015 and Thilagavathi et al., 2017). On the other hand, the bacterial titration by Colony Changing Unit (CCU) or by Colony Forming Unit (CFU) per dose takes long time (5-14days) (Stewke and Robertson, 1982).

Raviv et al. (2008) and Ehtisham et al. (2015) established a Real-time PCR assay that had an inherent quantitative nature using dual-labeled probe (Taqman) advantageous for microorganisms strain differentiation

owing to the superior sensitivity and improved specificity endowed by 3 hybridizing oligonucleotides (two primers and a probe)

The bacteriologicl examination and conventional PCR of MG live vaccine, that didn't have ability to differentiate between live vaccines, field strains) (Stewke and Robertson, 1982). This lack of ability to differentiate between the participating strains (F, ts11 and field strain) limits the level of control and the amount of information that could be gained from MG vaccine evaluation studies. Present study developed an alternative tool for the qualitative and quantitative differentiation between MG strains (F, Ts11 and field strain) in live and killed MG vaccine quality control especially challenge test and shedding determination that improve reliability and efficiency of the vaccine quality control studies in addition to strain differentiation.

MATERIALS AND METHODS

MG vaccine

Three types of commercial MG vaccinesused in this study, two live (vaccine F- strain and ts-11) and one killed vaccine.

MG field strain

It was obtained from the Reference Strain Bank at Central loboratory for Evaluation of Veterinary Biologics (CLEVB).

MG live vaccine and field strain titration

Live vaccines were reconstituted in the given manufacturer diluent. Vaccinesweрediluted ten–fold, different dilutions were streaked on pleuropneumonia like organism (PPLO) solid media. Starting dilution was 10^{10}CFU.Field strain was adjusted to10^{10}CFU and diluted ten-fold serial dilution.

rt-PCR for live MG vaccine (f strain and ts -11) and field strain :

Each ten dilution of two live MG vaccines and field strain ware centrifuged for 30 minutes, at 14,000 gandat 4°C. The supernatant was carefully removed and the pellet was suspended in 25 µl PCR grade water. The tube and the contents were boiled for 10 minutes and then placed on ice for 10 minutes before centrifugation at 14,000 gfor 5 minutes. Half amount of DNA extract (12.5 µl) of each vaccine dilution was tested by rt-PCR.The primers and labeled probes (FAM , HEX and ROX) are summarized in table 1 and according to Raviv et al. (2008).

rt-PCRwas performed in stratagene MX 3005P. Thermoprofilewas 95 °C for 15 min with optics OFF, and 40 cycles of 94 °C for15s followed by reaction specific primers and probe (Table 1). Annealing/extension temperatures for 60 s with optics ON. Cycle threshold number (CT value) was determined asthe PCR cycle number at which the fluorescence of thereaction crosses the florescence threshold. Any reaction with CT value was considered positive and any reaction without CTvalue, was considered negative. Standard curves were established according to Ehtisham et al. (2015). The quantitation and detection limit of each of the study's rt-PCRs were determined by one run of each concentration for MG live vaccines and field strain. Final results were doubled as half amount of DNA extract was used.

Experimental design

Four groups contained of thrity four Specific Pathogen Free chickens two weeks ago. Three groups were vaccinated with F-strain, ts -11 and killed vaccine as recommended by vaccine manufacturer and the forth group was kept separately as control. Three weeks of post vaccination, all groups were challenged with 0.5 ml containing 1×10^{8}CFU of overnight culture of MG field strain.

Air sac lesion scoring

The air sac lesion scoring was carried out at7 and 14 days post challenge to determine the level of protection. Ten birds from each of the experimental groups were necropsied at each three time 5, 7 and 14 days post challenge (DPC). The level of protection was evaluated by gross air sac lesion scoring on a scale from 0 to 4 air sac lesions examination. Also the protection rate was determined according to Whithear (1996) Protection rate = (protective vaccinated birds-protected un vaccinated bird)/ unprotected unvaccinated birds (Kleven et al., 1972).

Serological evaluating

10 Serum sample were taken from all group preandpost vaccination for four times (0, 5, 7, 14 DPC test). The serum samples were used for determining the level of the immune response by using MG ELISA antibody test kit (Synbioytics , Pro FLOK, Zoitis USA) (Javedet al., 2005; Zulfekar et al., 2015).

Shedding determination

Ten birds per each group were necropsied and sampled three times (5,7and 14 DPC).rt- PCR was carried out on laryngeal wash samples. Briefly, the larynx was cut at the base and put in 10 ml sterile plastic tubes filled with 5 ml Phosphate Buffer Saline (PBS), and vortexed for 30s. A 0.5 ml laryngeal wash solution was submitted for DNA extraction. The final results were powered 20× as 10% amount of laryngeal wash and half amount of DNA extract were used

Ethical aporoval

This study was approved procedures from Centeral laboratory for evaluation of veterinary biologics, Cairo, Egypt for humane handling of experimental animals.

RESULTS

Each reaction standard curve was determined by independent runs of each reaction using 10fold serial dilutions (10^{10}–10^{1} copies per reaction) of the reaction's

standard DNA control. The mean CT values, the linear equation and the R-squared value of the obtained standard curves are summarized in table 2 and figure 2. The minimal concentration of F-strain and ts-11, MG live vaccines were 10 and 10^3 CFU per sample respectively,while for field strain it was 10^2 CFU per sample as shown in table 2.

The rt PCR was highly specificity and differentiating for the target strain and gave negative to opposite strain as demonstrated in figure 1.

The protection rate was analyzed by air sac lesion, incase of f-strain vaccination frist group the healthy necropsied birds (no air sac lesion) were8,8 and 9 birds at 5, 7 and 14 DPC respectively, and for the ts-11 strain vaccination second group the healthy necropsied birds were 8, 7 and 8 birds at 5, 7 and 14 DPC respectively while incase of the killed vaccination third group , the healthy necropsied birds were 7, 6 and 8 birds at 5 , 7 and 14 DPC respectively but the positive control group 4 (non vaccinated group), the healthy necropsied birds were 1, 1 and 1 bird at 5, 7 and 14 DPC (Table 3). So the protection rate for F-strain and ts-11 stain and killed vaccine were 81.5%, 74% and 66.6 % respectively (Table 3).

As shown in table 4 the antibody titer against MG, F-strain, ts-11 strain and killed in sera were increased from 122 pre-vaccination level to 319, 259 and1009 at 3 weeks post vaccination and to 954, 763 and 1643 at 3 weeks postchallenge.

The four groups were sampled three times at 5D , 7D and 14DPC and the quantitative rt- PCR for different MG strains (F , ts -11 and field strain) was carried out on laryngeal wash. Vaccinated birds with F- strain vaccine demonstrated sharp decrease of the field strain count ($10^{5.7}$ and $10^{4.8}$CFU CFU) at 5DPC and 7DPC respectivel, then shedding was stopped at14DPC, but for F-strain was continuously shedding even at 14DFC. Vaccinated birds with ts-11 strain vaccine demonstrated decrease of the field strain count ($10^{8.1}$, $10^{7.1}$ and $10^{5.1}$CFU) at 5D, 7D and 14 DPC, butts-11 strain was continuously shedding. vaccinated birds with killed vaccine demonstrated slight decrease of field strain count ($10^{8.6}$, $10^{7.9}$ and $10^{5.8}$CFU) at 5D, 7D and 14 D post challenge also the shedding wasn't stopped (Table 4).

DISCUSSION

The evaluation of avian mycoplasma vaccines and the study of their mechanism of action as serological, protection rate and shedding determination have lacked the ability to differentially identify and quantify the participating strains within the vaccine quality control. The conventional PCR is not suitable for multi strain infection situations (Muhammad et al., 2017). The lack of ability to differentially identify the participating strains imposed significant limitations to the level of control that could be achieved in MG vaccines evaluation studies (Thilagavathi et al., 2017).

In this study The rt-PCR had ability to identify and quantify the two types of vaccines (F, ts-11 and Field strain) strains at the same reaction by using different labeled probe (FAM, HEX and ROX) respectively. The sensitivity (minimal CFU that gave positive results) of the rt PCR for F-strain, ts-11 and field strain were 10 , 10^3and10^2CFU / sample respectively (table 2). Ehtisham et al. (2015) detected 10^2CFU MG / sample using rt-PCR taqman labeled probe while Raviv et al. (2008) detected 6.5×10^1 CFU MG / sample**.**

the antibody titer against MG, F-strain, ts-11 strain and killed in sera were increased from 122 pre-vaccination level to 319, 259 and1009 at 3 weeks of post vaccination and to 954, 763 and 1643 at 3 weeks of postchallenge. The birds taken killed vaccine apparently gave immune response than two live vaccine (F-strain and ts-11).The results were similar to the results of Avakian et al. (1988) and Pakpinyo et al. (2014).

The protection rate for birds vaccinated with F-strain vaccine was higher (81.5%) than ts-11 (74) and killed vaccine (66.6%) against field strain. This result was similar to the results of Jacob et al. (2014) and Jacob et al. (2015).

Regarding the F-strain live vaccine had ability to stop the shedding of the field strain at 14 DPC. On the contrary the ts-11 and killed vaccine didn't have ability to stop the shedding till at 14 DPC. Moreover, Raviv et al. (2008) recorded that birds vaccinated with 6/85 and K5831 strain live vaccine demonstrated a stopping the shedding of challenge strain.

Results of molecular assay showed that the ability to differentiate between a known array of Mycoplasma strains (F, ts-11 and field strain) in a mixed sample. The rt-PCR with dual-labeled probe technology endowed the method with its superior sensitivity, specificity and quantitative properties. The initial application of this quantitative strain differentiating tool was designed for live and killed mycoplasma vaccine quality control and indeed provided a significant upgrade to this area of research. The demonstrated concept of differential rt-PCR is general and could be considered for a variety of research applications in mycoplasmology and microbiology.

Table 1. The primer and labeled probe (FAM and HEX) rt- PCR specifications of *mycoplasma*live vaccine and field strain

Types of mycoplasma Strains	Gene and GenBank sequence accession #	Forward (F) primer sequence (5-3)	Reverse (R) primer sequence (5- 3)	Probe (P) Sequence (5-3)	Type of fluorescence	Oligos location on GenBank sequence	PCR product size (bps)	Anneling/eextentiontemputure
F strain	mgc2, AY556230	gttcaagaaccaactcaacca	Gattaagaccgaattgtggattg	caaccaggatttaatcaacctcag	FAM	F: 217–237 R: 328–306 P: 280–303	112	61 ℃
ts-11 strain	mgc2 AY556232	ctcaagaaccaactcaacca	Ggggattaggaataaattgcggat		HEX	F: 218–237 R: 331–308 P: 280–303		
Field strain	pvpA, AY556306	ttctcaaccacgcccaatg	ggttagatccaccaactccca	Caatgggtgctccaaatcctcaac	ROX	F: 246–264 R: 364–344 P: 290–313	119	61 ℃

FAM, HEXandRox were fluorescence dye

Table 2. Summary of the mean CT values, the linear equations and the R-squared values of the rt- PCRs for F-strain ,ts-11 and field strain

Dilution (CFU/ sample)	F-strain	ts-11	Field strain
10^{10}	11.21	13.41	13.1
10^{9}	14.59	16.9	16.4
10^{8}	17.25	20.17	19.17
10^{7}	20.91	23.46	22.77
10^{6}	24.69	26.79	24.98
10^{5}	27.55	30.36	29.01
10^{4}	31.71	33.98	32.18
10^{3}	32.68	39.09	37.26
10^{2}	33.98	Negative	38.77
10	39.09	Negative	Negative
Linear equation	**Y=** -0.3219X+13.691	**Y=** -0.305X+14.167	Y=-0.3136X+14.0833
R-squared	**0.9877**	**0.9811**	**0.9911**

Table 3. The summary of the airsac lesion and the protection rate for F-strain, ts-11 and killed *Mycoplasma gallisepticum* vaccine

Group	Group 1 (F- strain)			Group 2 (ts-11)			Group 3(killed vaccine)			Group 4 (Positive control)		
Day post challenge	5	7	14	5	7	14	5	7	14	5	7	14
Protected bird /total number	8/10	8/10	9/10	8/10	7/10	8/10	7/10	6/10	8/10	1/10	1/10	1/10
	25/30			23/30			21/30			3/30		
Protection rate*	**81.5%**			**74%**			**66.6%**			0		

*Protection rate = (protective vaccinated birds-protected un vaccinated bird)/ unprotected unvaccinated birds

Table 4. Serological evaluation (ELISA antibodies mean titer) for F-strain, ts-11 and *Mycoplasma gallisepticum* killed vaccine

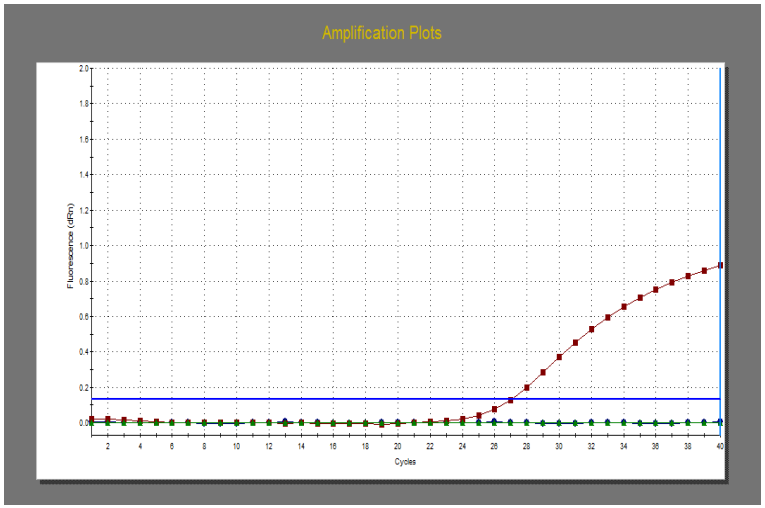
Items	Group 1 (F- strain)	Group 2 (ts-11)	Group3 (killed vaccine)	Group 4 (Positive control)
0 day of vaccination	122	122	122	122
3 weeks of post vaccination	319	259	1009	113
first week of post challenge	706	543	1203	116
second week of post challenge	811	546	1508	234
third week of post challenge	954	763	1643	467

Table 5. Determination of the amount of microbial shedding for the F-strain , ts-11, and killed *Mycoplasma gallisepticum*vaccineafter 5, 7 and 14 days post challenge

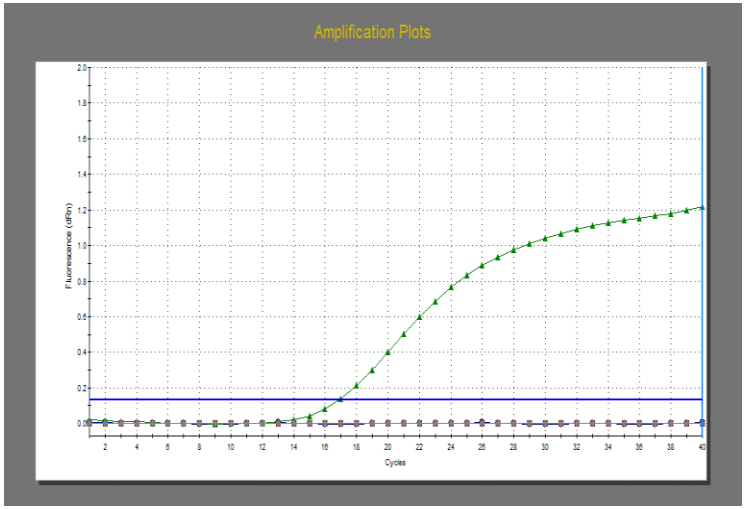
		Group 1 (F- strain)			Group 2 (ts-11)			Group3 (killed vaccine)			Group 4 (Positive control)		
Type of Probe fluorescence		FAM (F-strain)	HEX (Ts-11)	ROS (Challenge strain)	FAM (F-strain	HEX (Ts-11	ROS (Challenge strain)	FAM (F-strain)	HEX (Ts-11)	ROS (Challenge strain)	FAM (F-strain)	HEX (Ts-11)	ROS (Challenge strain)
Linear equation		**Y=** -0.3219X +13.691	**Y=** -0.305X +14.167	Y=-0.3136X +14.083	**Y=** -0.3219X +13.691	**Y=** -0.305X +14.167	Y=-0.3136X +14.083	**Y=** -0.3219X +13.691	**Y=**-0.305X +14.167	Y=-0.3136X +14.083	**Y=** -0.3219X +13.691	**Y=** -0.305X +14.167	Y=-0.3136X +14.083
Mean Ct for 10birds	5 DPC	22.2	No Ct	30.3	No Ct	26.4	23.1	No Ct	No Ct	21	No Ct	No Ct	22.1
	7 DPC	23.6	No Ct	33.2	No Ct	33.9	25.9	No Ct	No Ct	23	No Ct	No Ct	20.7
	14 DPC	27.9	No Ct	No Ct	No Ct	37.8	32.2	No Ct	No Ct	30	No Ct	No Ct	22.9
Mean titer for 10 birds*	5 DPC	$10^{7.7}$	**-ve**	$10^{5.7}$	**-ve**	$10^{7.3}$	$10^{8.1}$	**-ve**	**-ve**	$10^{8.6}$	**-ve**	**-ve**	$10^{8.2}$
	7 DPC	$10^{6.2}$	**-ve**	$10^{4.8}$	**-ve**	$10^{4.9}$	$10^{7.1}$	**-ve**	**-ve**	$10^{7.9}$	**-ve**	**-ve**	$10^{8.6}$
	14 DPC	$10^{5.8}$	**-ve**	**-ve**	**-ve**	$10^{3.7}$	$10^{5.1}$	**-ve**	**-ve**	$10^{5.8}$	**-ve**	**-ve**	$10^{7.9}$

*The titer was powered 20 × as 10% amount of laryngeal wash and half amount of DNA extract were used; Ct: cycle threshold value. DPC = days post challenge. FAM, HEXandRox were fluorescence dye

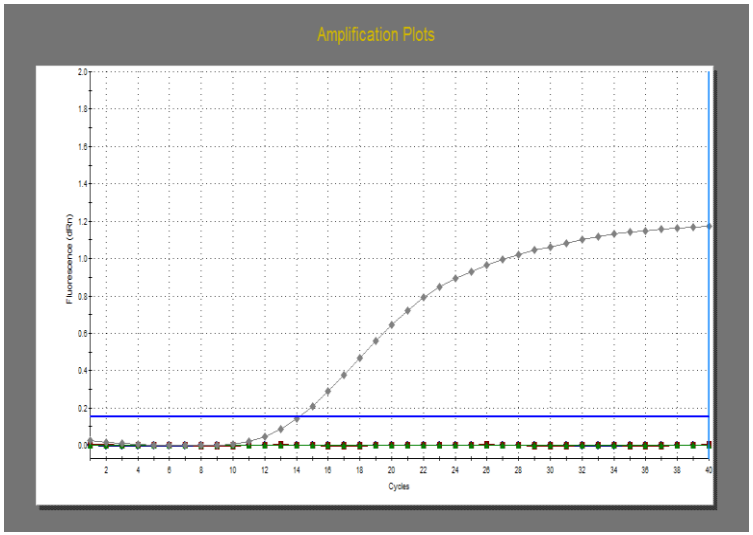
Figure 1. Specificity test for differentiation between different F- strain ,ts -11 and field *Mycoplasma gallisepticum* strain



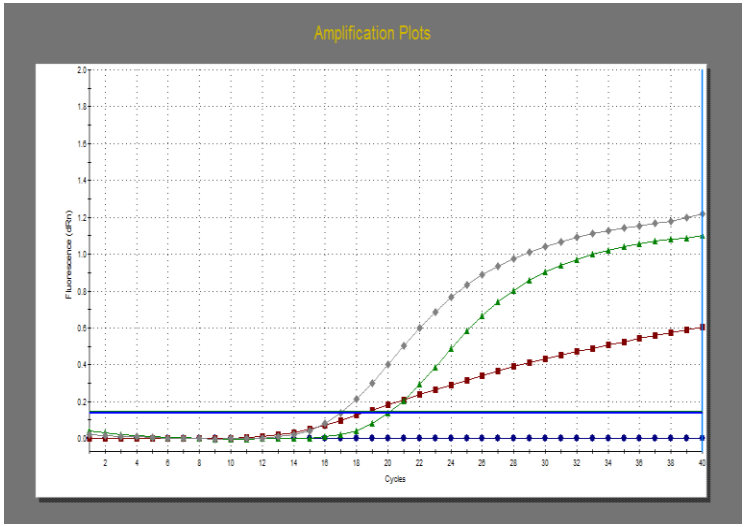
A



B



C



D

- **Run A:** the sample contain DNA extract from Fstrain was carried out using primers and probs for F-strain (red) , ts-11 (green) and field strain (gray) and gave signal for F-strain only.
- **Run B:** the sample contain DNA extract from ts-11 was carried out using primers and probs for F-strain (red) , ts-11 (green) and field strain (red) and gave signal for ts -11 strain only.
- **Run C:** the sample contain DNA extract from challenge strain was carried out using primers and probs for F-strain (red) , ts-11 (green) and field strain (red) and gave signal for field strain only.
- **Run D (two samples):** the sample no. 1 contain mix DNA extract from F-strain (red) , ts-11 (green) and field strain (red) was carried out using all primers and probs and gave signals for all strains. On the anther hand the sample no 2 contain normal saline thatno gave signal (blue).

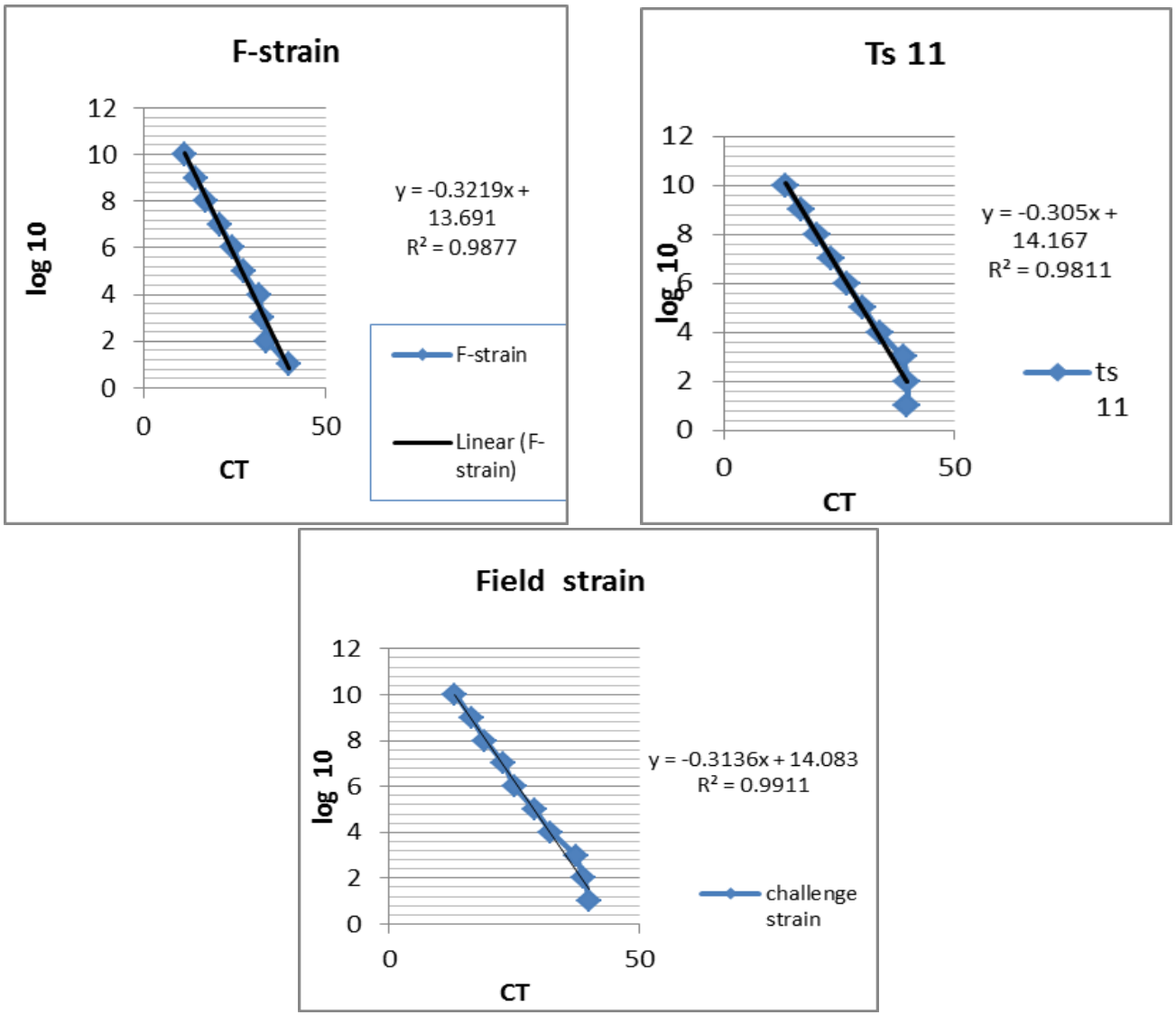


Figure 2. Standard curve and the calculation equation for F-strain , ts-11, and field *Mycoplasma gallisepticum* strain**s** Log10 = 10^2, 10^3, 10^4etc. CT = Cycle threshold

DECLARATIONS

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors participated in making the design,performing the experiment, analyses of the data, and writing the paper.

REFERENCES

Avakian AP, Kleven SH and Glisson JR (1988). Evaluation of the specificity andsensitivity of two commercial enzyme-linkedimmunosorbent assay kits, the serum plateagglutination test, and the hemagglutination-inhibition test for antibodies formed in responseto*Mycoplasma gallisepticum*. Avian Disease, 32(2): 262 - 272. DOI:https://doi.org/10.2307/1590813

Burnham MR, Branton SL, Peebles ED, Lott BD and Gerard PD (2002). Efeects of F- strain *Mycoplasama gallisepticum* inoculation at twelve weeks of age on performance and egg characteristics of commercial egg-laying hans, Poulty Science, 81: 1478-1485. DOI:https://doi.org/10.1093/ps/81.10.1478

Ehtisham S, Rahman SU, Khan MI, Younus MM and Nasair A (2015).A simplified duplex real-time PCR incorporating TaqMan minor groove binder (MGB) probes and an exogenous internal positive control for the simultaneous detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* cultures. Veterinarni Medicina, 60(5): 268–273. DOI:https://doi.org/10.17221/8179-vetmed

Feberwee ADR, MekkesJJ, de WitEG, Hartman and Pijpers A (2005). Comparison of Culture, PCR, and Different Serologic Tests for Detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* Infections. Avian Diseases, 49(2): 260-268. DOI:https://doi.org/10.1637/7274-090804r

Jacob R, Branton SL, Evans JD, Leigh SA and Peebles ED (2014). Effects of live and killed vaccines against Mycoplasma gallisepticum on the performance characteristics of commercial layer chickens. Poultry Science, 93(6):1403-9. DOI:https://doi.org/10.3382/ps.2013-03748

Jacob R, Branton S L, Evans JD, Leigh SA and Peebles ED (2014). Effects of different vaccinecombinations against Mycoplasama gallisepticum on the internal egg and eggshell characteristics of commercial layer chicken. Poultry Science, 94(5):912-7. DOI:https://doi.org/10.3382/ps.2008-00099

Javed MA, Frasca S, Rood JR, Cecchini K, Gladd M, Geary SJ and Silbart LK (2005). Correlates of immune protection in chickens vaccinated with Mycoplasma gallisepticum strain GT5 following challenge with pathogenic M. gallisepticum strain R low. Infectious Immunity, 73(9): 5410–5419. DOI:https://doi.org/10.1128/iai.73.9.5410-5419.2005

Kleven SH, King DD and Anderson DP (1972). Airsaculitis in broilers from Mycoplasma synoviae: effect on air-sac lesions of vaccinating with infectious bronchitis and Newcastle virus. Avian Disease, 16: 915–924. DOI:https://doi.org/10.2307/1588772

Ley DL (2008).Mycoplasma gallisepticum infection. In Diseases of Poultry, pp. 807–834.Edited by Y M Saif, A M Fadly, J R Glisson, L R McDougald, L. K. Nolan and D. E. Swayne. Ames, IA: Blackwell. DOI:https://doi.org/10.1016/b978-0-7020-2862-5.x5001-6

Mettifogo E, Melissa Buzinhani, Marcos RBuim, Jorge Timenetsky and Antonio JPiantino Ferreira (2015). Evaluation of a PCR multiplex for detection and differentiation of Mycoplasmasynoviae, M. gallisepticum, and M. gallisepticum strain F-vaccine. Veterinary Brasilia 35(1):13-18. DOI:https://doi.org/10.1590/s0100-736x2015000100004

Muhammad F, Syed KF, Urooj Z, Taseer AK and Aqeel A (2017). Development and Evaluation of CultureEnhanced Tetra-PCR for Differential Diagnosisof *Mycoplasma gallisepticum* and *M. synoviae. Pakistan* Journal Zoology, 49(6): 2133-2140. DOI://dx.doi.org/10-17582/journal.pjz/2017.49.6.2133.2140.

Moraes ME, Pereira GBA, Astolfi-Ferreira C S and Ferreira AJP (2013). In fecção experimental por Mycoplasma gallisepticume Escherichia coli emperus. Pesq. Veteriary Brasilia, 33:975-978. DOI:https://doi.org/10.1590/s0100-736x2013000800004

OIE (2013). OIE Quality Standard and Guidelines for Veterinary Laboratories: Infectious Diseases. Office Internationale des Epizooties, Paris, France. DOI:https://doi.org/10.20506/rst.issue.32.2.53

Pakpinyo S, Pitayachamrat P, Saccavadit S, Santaswang T, Tawatsin A and Sasipreeyajan J (2014). Laboratory Diagnosis of Mycoplasma gallisepticum(MG) Infectionin Experimental Layer ChickenReceiving MG Vaccines and MG Organisms.Journal of veterinary medicine, 36(2): 29-37. DOI:https://www.tci-thaijo.org/index.php/tjvm/article/view/36322

Raviv Z, Scott A, Callison N, Noel F and Kleven S (2008). Strain differentiating real-time PCR for Mycoplasma gallisepticum live vaccine evaluation studies. Veterinary Microbiology, 129: 179–187. DOI:https://doi.org/10.1016/j.vetmic.2007.11.017

Stewke GM and Robertson JA (1982).Comparison of Two Methods For Enumeration of Mycoplasmas. Journal of

clinical microorganism, 959-961. DOI:https://doi.org/10.1049/wis.1982.0007

Thilagavathi K, Sivaseelan S, Balasubramaniam GA, Balasurbramaniam A, Arulmozhi A and Madheswaran R (2017). Detection of Mycoplasma gallisepticum from field samples of laying chicken using PCR. International Journal of science environment and technology, 4(6): 2594-2499. DOI:https://doi.org/10.9775/kvfd.2014.12505

Whithear KG (1996).Control of avian mycoplasmoses by vaccination. Rev Science Technolongy Off. IntEpiz, 15: 1527–1553. DOI:https://doi.org/10.20506/rst.15.4.985

Whithear KG, HarringanKE andGhiocas E (1990). Safety of temperature sensitive mutant Mycoplasma gallisepticum vaccine, Australia veterinary journal, 67: 159-165. DOI:https://doi.org/10.1111/j.1751-0813.1990.tb07745.x

Yoder WH (1990). Yoder Jr., Avian Mycoplasmosis, Diagnostic Procedures in Veterinary Bacteriology and Mycologyedition, academic press,San Diego, Calif, USA, 5th edition, 1990, diagnostic procedures in veterinary bacteriology and mycology edition, academic press, San Diego, Calif, USA, 5th edition, 1990. DOI:https://doi.org/10.1016/0378-1135(85)90068-9

Zulfekar A, Mostafizer R and Sultana S (2015). Seroprevalence of Mycoplasma gallisepticum antibody by ELISA and serum plate agglutination test of laying chicken. Veterinary World, DOI: 10.14202/vetworld.2015.9-14