

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

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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 fourth group was considered control. Challenge test was applied by 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 7 days 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 (5-14 days) (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 bacteriological 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 vaccines used 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 laboratory for Evaluation of Veterinary Biologics (CLEVB).

MG live vaccine and field strain titration

Live vaccines were reconstituted in the given manufacturer diluent. Vaccines were diluted ten-fold, different dilutions were streaked on pleuropneumonia like organism (PPLO) solid media. Starting dilution was 10^{10} CFU. Field strain was adjusted to 10^{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 were centrifuged for 30 minutes, at 14,000 g and at 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 g for 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-PCR was performed in Stratagene MX 3005P. Thermoprofile was 95 °C for 15 min with optics OFF, and 40 cycles of 94 °C for 15s 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 as the PCR cycle number at which the fluorescence of the reaction crosses the fluorescence threshold. Any reaction with CT value was considered positive and any reaction without CT value,

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 thirty 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 fourth 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 at 7 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 unvaccinated bird) / unprotected unvaccinated birds (Kleven *et al.*, 1972).

Serological evaluating

10 Serum samples were taken from all group pre and post 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 (Synbioyitics, Pro FLOK, Zoetis USA) (Javed *et al.*, 2005; Zulfekar *et al.*, 2015).

Shedding determination

Ten birds per each group were necropsied and sampled three times (5, 7 and 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 approval

This study was approved procedures from Central 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 10 fold 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, in case of f-strain vaccination first group the healthy necropsied birds (no air sac lesion) were 8, 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 in case 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 strain 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 and 1009 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) at 5DPC and 7DPC respectively, then shedding was stopped at 14DPC, but for F-strain was continuously shedding even at 14DPC. 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, but ts-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, 10^3 and 10^2 CFU / sample respectively (table 2). Ehtisham et al. (2015) detected 10^2 CFU 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 and 1009 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 mycoplasma 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/extension temperature
F strain	mgc2, AY556230	gttcaagaaccaactcaacca	Gattaagaccgaattgtg gattg	caaccaggattta	FAM	F: 217–237 R: 328–306 P: 280–303	112	61 °C
ts-11 strain	mgc2 AY556232	ctcaagaaccaactcaacca	Ggggattaggaataaat tgcggat	atcaacctcag	HEX	F: 218–237 R: 331–308 P: 280–303		
Field strain	pvpA, AY556306	ttctcaaccacgcccaatg	ggtagatccaccaactc cca	Caatgggtgctcc aaatcctcaac	ROX	F: 246–264 R: 364–344 P: 290–313	119	61 °C

FAM, HEX and Rox 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

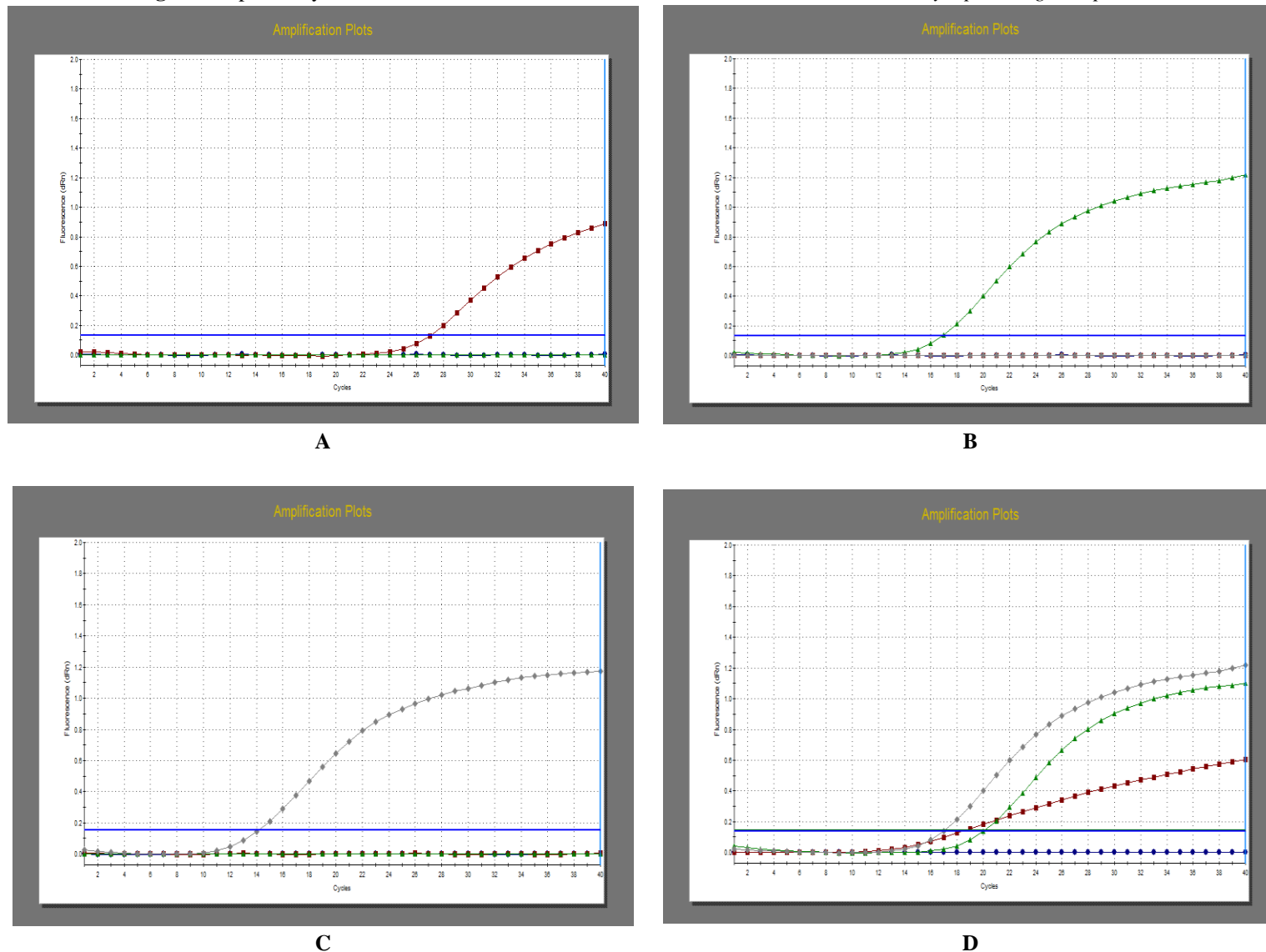
Items	Group 1 (F- strain)	Group 2 (ts-11)	Group3 (killed vaccine)	Group 4 (Positive control)
0 day of vaccination	122	122	122	122
3 weeks of post vaccination	319	259	1009	113
first week of post challenge	706	543	1203	116
second week of post challenge	811	546	1508	234
third week of post challenge	954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

Table 5. Determination of the amount of microbial shedding for the F-strain , ts-11, and killed *Mycoplasma gallisepticum* vaccine after 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, HEX and ROS were fluorescence dye

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



- **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 another hand the sample no 2 contain normal saline thatno gave signal (blue).

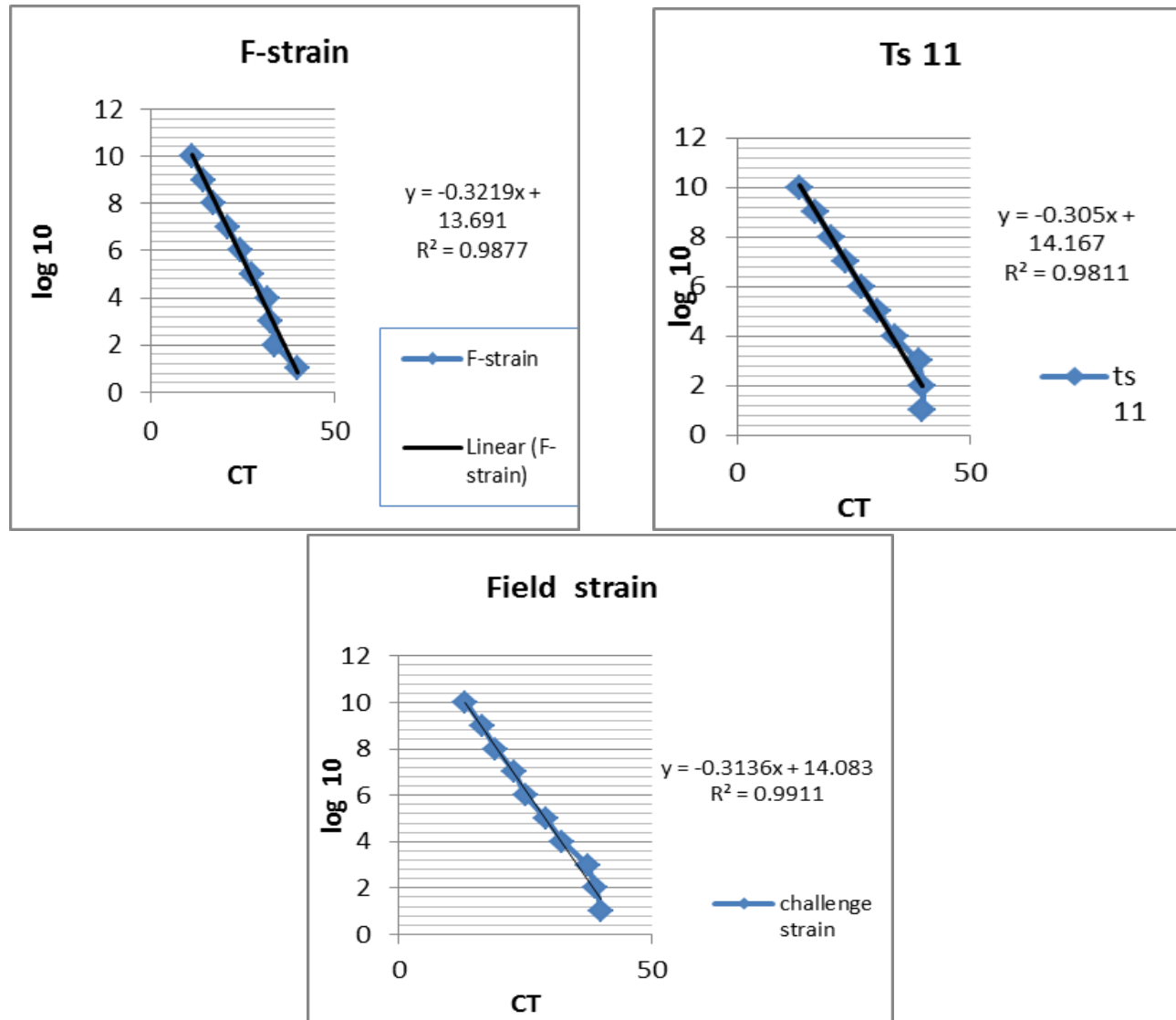


Figure 2. Standard curve and the calculation equation for F-strain , ts-11, and field *Mycoplasma gallisepticum* strains
Log10 = 10², 10³, 10⁴etc. CT = Cycle threshold

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.

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