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Use of Real Time PCR and Semi Quantitative Lateral Flow Chromatography Test for Assessment of Live *Mycoplasma gallisepticum* Vaccine Efficacy In Chicken

Rafik H. Sayed, Nermeen Mohamoud, Sahar M. Saber and Abdelhakim M. Ali

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

Abstract: *Mycoplasma gallisepticum* (MG) is the most pathogenic and economically important pathogens that cause respiratory disease and production losses in poultry. MG live vaccine is an approach to reduce the susceptibility to infection and to prevent economic losses. The goal of this study was to develop an alternative methods for evaluation of live vaccine usually required to reduce the time and effort such as quantitative real time PCR (rt-PCR) and lateral flow chromatography (LFC) comparing to the traditional methods. About ten batches of mycoplasma vaccines were tested. rt-PCR assays were implemented for titration and identity of two types of live MG vaccine (F and ts-11 strain), on the other hand the LFC detect the quantity of mycoplasma in all batches tested of all live mycoplasma vaccine but not identify types of it. There was no significance different between rt-PCR count and bacteriological count (CFU) of live MG vaccine, *P* value was 0.01 and 0.012 respectively. The minimal CFU MG that gives positive for rt –PCR was 10 CFU but in case of prepared LFC test was 10^3 CFU.

Key words: *Mycoplasma gallisepticum* · Real time PCR lateral flow chromatography

INTRODUCTION

Mycoplasma gallisepticum (MG) is an important pathogen of poultry worldwide, causing chronic respiratory disease in chickens and turkeys, reduction in egg production and considerable economic losses to the poultry industry. These losses results from condemnation or downgrading of carcasses, reduced feed and egg production efficiency, .Increased medication costs are factors that make costliest disease problems confronting [1]. Live MG vaccines appear to be more effective and therefore more popular than bacterin [2, 3]. An important characteristic of MG live vaccines is their ability to increase resistance to wild-type strain infection and to displace wild-type strains with the vaccine strain on multiple-age production sites [4, 5].

The quality control of live MG vaccine was depended 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. On the other hand, the bacterial titration by colony changing unit (CCU) or by colony forming unit (CFU) per dose take a long time for (5-14 days) [6].

The lateral flow chromatographic technique is a simple strip or device assays, which gain more and more popularity as a rapid diagnostic method that can be used for direct diagnosis at the production line or in the field in additionally used for semi quantitative methods. This technique is among the most widely used technique for detection of microbial analyte in clinical specimens such as *Vibrio harveyi* [7], *Salmonella enteric sub sp. Enterica serovar typhimurium* (*Salmonella typhimurium*) [8], *S. aureus* [9], *Yersinia pestis* in human [10], *Streptococcus suis* serotype 2 [11], *Tilletiaindica* [12], *Leptospira* in urine [13], *Vibrio cholera* [14], This test minimizes the titration of MG live vaccine to 5 minutes but it does not differentiate between the two types of live vaccines.

So in this work an alternative method were established to increase the sensitivity, specificity and accuracy of the identity test and titration test such as rt- PCR with two labaled probes to differentiate between two types of MG live vaccine (F and ts-11) at the same time within 2 hrs [15].

Corresponding Author: Rafik H. Sayed, Central Laboratory for Evaluation of Veterinary Biologics (CLEVB, ARC) Abbasia, Cairo, Egypt. E-mail: rafikhamed1010@hotmail.com.

MATERIALS AND METHODS

Live Vaccine MG [6]: The two types of live MG vaccine used in this study, F- strain (Schering-Plough) and ts-11 (Vaxsafe MG, Bioproperties) to establish the rt-PCR standard curve and linear equation, Plus the ten batches of each type vaccines were evaluated tested by the rt-PCR and LFC.

Vaccine Titration: The vaccine after being reconstituted in the diluent given by the manufacturer, It was re-diluted to one dose. The one dose was diluted to ten folder dilution. 0.1 ml of each dilution was streaked on PPLO solid media (colony forming unit (CFU) and put in closed containers then incubated in a humid incubator under low CO2 atmosphere tension at 37°C for 3-14days. The plates were examined under low magnification using a dissecting microscope. Mycoplasma colonies are usually observed within 3-7 days and appear as a tiny, smooth colonies 0.1 to 1 mm in diameter with dense, elevated center giving the colonies "Fried Egg" appearance colonies in each dilution were counted according to Read and Muench [16].

The rt –PCR for live MG vaccine (F strain &ts11) [15]: The two vaccines were adjusted to contain 10^{10} CFU and then diluted for ten serial dilution. Each dilution was centrifuged for 30 minutes at 14, 000 *g* 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 [17]. Half amount of DNA extract (12.5 µl) of supernatant. Each vaccine dilution was tested by rt-PCR.

Each reaction was performed in Stratagene MX 3005P using a thermocycler program of 95°C for 15 min with optics OFF; and 40 cycles of 94°C for 15s followed by the reaction specific annealing/extension temperatures as specified (Table 1) for 60 s with optics ON. The cycle threshold number (CT value) was determined to be the PCR cycle number at which the fluorescence of their action. Any reaction recorded CT value, it was considered positive and any reaction did not record CT value, it was considered negative.

Standard curves were established. The quantitation and detection limit of each of the study's real-time PCRs were determined by one run of each concentration for MG live vaccines and determination of linear equation. The final results were multiplied double as half amount of DNA extract was used.

Specificity Test: Each primers and probe of bath types of mycoplasma were run reaction for opposite to each other and with another bacteria (*E. coli Escherichia coli*, *Klebsilla pneumonia*, *Pseudomonas aerguinosa*, *B. cereus Bacillus cereus and S. auraus*).

Lateral Flow Chromatography Test (LFC)

Production of Polyclonal Antibodies in Guinea Pigs and Rabbit.: Antisera of mycoplasma were prepared in rabbit and guinea pigs according to Gulbenkian *et al*.[18].

Concentration and purification of polyclonal antibodies of guinea pig and rabbit polyclonal using the ammonium sulphate procedure [19].

The concentration of purified antibody was measured by spectrophotometer.

Preparation of Colloidal Gold Nanoparticles [20]: One ml of 1% (m/v) sodium citrate solution was added to 100ml boiled deionized water. When the mixture was heated to boiling again, one ml of 1% (m/v) Gold chloride (HAuCl4) solution was added rapidly by constant stirring. After the color of the solution changed to wine red (in about 2min), the solution was boiled for another 10 min. After cooling, deionized water was added until the volume reached 100 ml. The obtained gold colloid was supplemented with 0.02% (m/v) of sodium azide and stored at 4°C. The particle diameter was checked with transmission electron microscopy (TEM, H-7650).

Conjugation of Rabbit Polyclonal Antibodies and Colloidal Gold [21]: The pH of colloidal gold solution was adjusted to 8.5 with 0.02Mole potassium carbonate (K_2CO_3) with gentle stirring, 0.1ml of rabbit purified poly clonal antibody (2mg/0.1ml, in 0.05% NaCl buffer) was added drop wise to 10ml of pH-adjusted colloid gold solution. The mixture was gently mixed for 10 min, blocked with Poly Ethylene Glycol (PEG) [20, 000, 1% (m/v) final concentration] by stirring for another 15 min and centrifuged at 10, 000 g for 30 min. The gold pellets were suspended in1mL dilution buffer [20mM Tris/HCI buffer (pH 8.2) containing 1% (w/v) bovine serum albumin (BSA), 3% (w/v) sucrose and 0.02% sodium azide] and stored at 4°C until used.

Preparation of lateral flow chromatography test [22]:

- Sample pad: a glass fiber was saturated with phosphate buffered saline (PBS) solution (pH 7.2) containing 0.3% Tween-20 and 0.5% (w/v) triton X100 and dried at 37°C and kept under dry conditions at room temperature until used.

Table 1: Summary of the primer and labeled probe (FAM and HEX) rt- PCR specifications of MG live vaccine:

Types of MG livevaccine	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)	Ann/Ext. temp.
F strain	mgc2, AY556230	gttcaagaaccaactcaacca	gattaagaccgaattgtggattg	caaccaggatttaatcaacctcag	FAM	F: 217–237 R: 328–306 P: 280–303	112	61 °C
ts-11	mgc2 AY556232	ctcaagaaccaactcaacca	ggggattaggaataaattgcggat		HEX	F: 218–237 R: 331–308 P: 280–303		

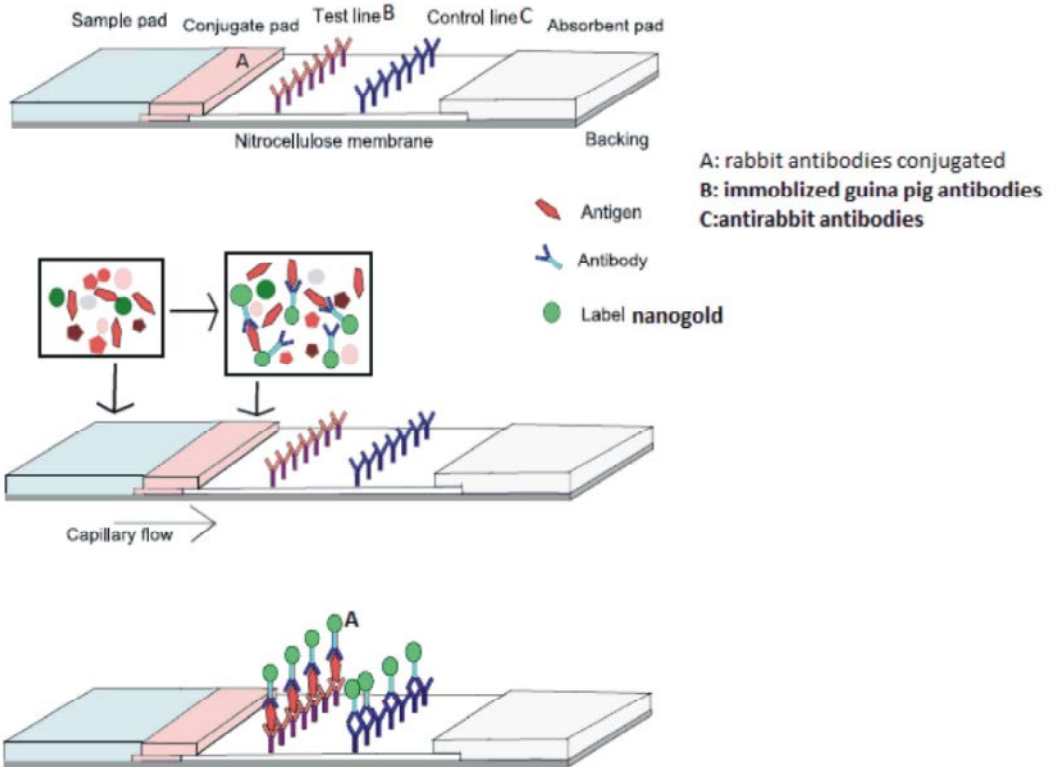

Fig. 1: The principle of lateral chromatography test

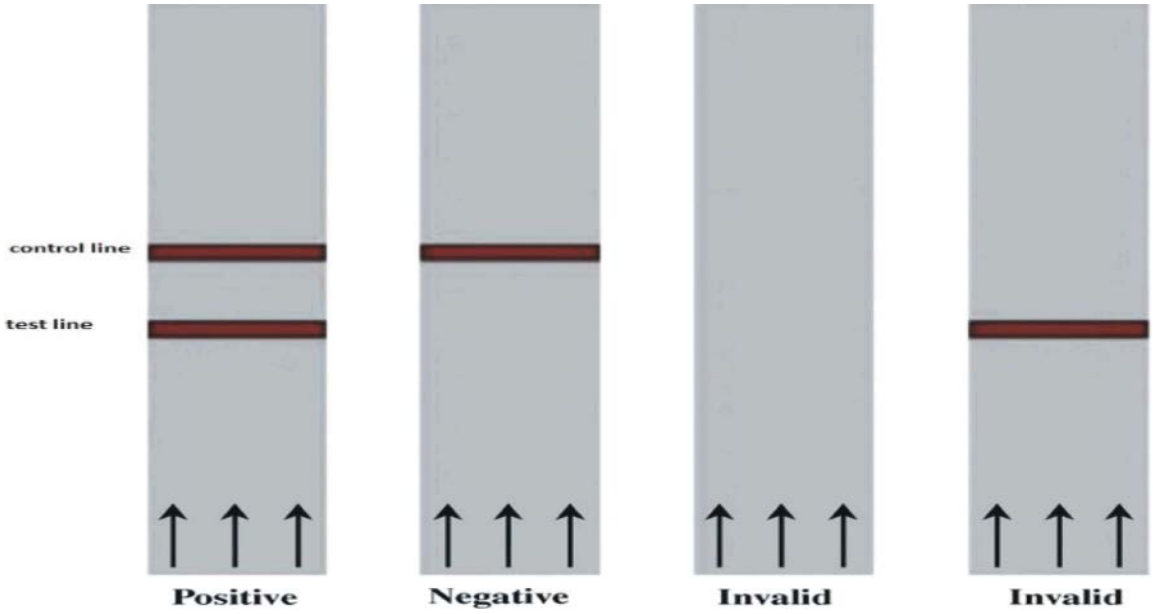

Fig. 2: The interpretation of the results of the lateral chromatography test

- The conjugate pad: It was prepared as follows: a glass fiber was treated with 0.1% Tween-20 for 10 min and dried at 60°C. The prepared glass fiber was cut into sections (4cm×0.5 cm) and then saturated with 0.15mL of colloidal gold probe. The conjugate pad was dried for 1 h at 37°C and stored under dry conditions at 4°C until used.
- Nitrocellulose membrane: the dispenser (BIODOT XYZ-3) was used to dispense two lines on the NC membrane (25mm×300 mm). Guinea pigs a polyclonal antibody (1.5mg/ 0.1ml) was dispensed around the bottom a the test line(1µL per 1 cm line) while anti-rabbit IgG ($1mgmL^{-1}$) was dispensed at the upper position as the control line (1µL per 1 cm line). The distance between two lines was 5mm. After the applying of the test line, the membrane was dried for 2 to 6 hours in room temperature. Then it was blocked with a membrane blocking buffer by immersing the membrane into the buffer. After the whole membrane was wetted, it was washed by immersing it five times in the first PBS and 5 times in the second PBS solution. After that, the membrane was covered with top laminate and cut into 0.5-cm-width test-strips by using an automated cutter machine as shown in Fig. (1).

Two red bands at the test and control zones are developed with no further addition of reagent. If the MG with concentration below the detection limit, only one band at the control zone is visualized. If no band developed at both zones, the test is invalid (Fig. 2). The intensity of the test line is in proportion to the amount of MG present in the sample. The control zone acts as a positive control to assure that functional, conjugated antibody migrated throughout the system. The total assay time is less than 5 min. The estimation of the test-strip results can either be performed visually with the naked eye.

Specificity Test: Pure culture of various bacterial culture (*E. coli*, *Klebsilla pneumonia*, *Pseudomonas aerguinosa*, *B. cereus and S. auraus)* were tested with LFC.

Sensitivity Test of LFC: The lowest dilution of mycoplasma detection by this test measure the sensitivity of it.

Application of rt-PCR and Lfc on Mycoplasma Vaccine Batches: About 10 batches of each of mycoplasma F and ts-11 living vaccines were test by both pervious tests and compared with traditional test. The final results were analyzed by IBM SPSS statistics 21 using Paired Samples Statistics and correlations test.

RESULTS

Each dilution of each Live vaccine was running at the same reaction (amplification plots, Figure 3). The minimal concentration of F- strain MG live vaccine was 10^1 CFU per sample , while for ts-11 MG live vaccine it was 10^3 CFU per sample as shown in Table (2).

The mean Ct values, the linear equation and r-squared value of the obtained standard curves of two types of MG live vaccine were summarized in table (2) and Figure (4).

The reaction was negative for opposite type of MG live vaccine and another bacterial type.

The minimal concentration of MG live vaccine that gave positive were 10^3 CFU but this test cannot be differentiate between the two types of vaccine as shown Figure (5).

The reaction was positive for opposite type of MG live vaccine but give negative for another bacterial type.

The ten batches of each live MG vaccine (F- strain & ts-11) were titrated by rt- PCR and gave results similar to bacteriological titration and there was no significance difference P=0.02 for F- strain and P=0.01 for ts-11 as shown in Table (3). The P value was considered significant between (0.01-0.05).

In case of LFC test the results was same in the sensitivity test of it figures (5&6) but there was no differentiation and identity of the two types of live MG vaccines.

Table 2: Summary of the mean CT values, the linear equations and the R-squared values of the real-time PCRs for F-strain and ts-11 (FAM & HEX) live MG vaccines

Dilution (CFU/ sample)	F- strain	Ts-11
10^{10}	11.21	13.41
10^9	14.59	16.9
10^8	17.25	20.17
10^7	20.91	23.46
10^6	24.69	26.79
10^5	27.55	30.36
10^4	31.71	33.98
10^3	32.68	39.09
10^2	33.98	Negative
10	39.09	Negative
Linear equation	Y= -0.3219X+13.691	Y= -0.305X+14.167
R-squared	0.9877	0.9811

Table 3: The comparison between the rt-PCR titer and bacteriological count for F-strain and ts-11 live MG batches vaccines:

F- strain					Ts-11				
Batch no.	Ct	Equation	rt-PCR titer	Mycoplasma count	Batch no.	Ct	Equation	rt-PCR Titer	Mycoplasma count
1	24.2	Y= -0.3219X+13.691	$9X10^5$	$7X10^5$	1	26.04	Y=- 0.305X+14.167	$2x10^6$	10^6
2	24.51	The concentration doubled	$8X10^5$	$8X10^5$	2	26.73	The concentration doubled	$1X10^6$	$9X10^5$
3	24.45	because the half amount of	$8X10^5$	$8x10^5$	3	26.01	because the half amount of	$2x10^6$	10^6
4	25.11	DNA extracted was tested	$6X10^5$	$6X10^5$	4	27.1	DNA extracted was tested	$9X10^5$	$8x10^5$
5	25.14		$5X10^5$	$4X10^5$	5	28.04		$6X10^5$	$6X10^5$
6	24.43		$8X10^5$	$6X10^5$	6	26.11		$2x10^6$	$1X10^6$
7	23.99		$9X10^5$	$8X10^5$	7	27.78		$6X10^5$	$6X10^5$
8	24.44		$8X10^5$	$8X10^5$	8	28.01		$6X10^5$	$5X10^5$
9	23.33		$2x10^6$	$1X10^6$	9	26.63		$1X10^6$	$1X10^6$
10	24.07		$9X10^5$	$8X10^5$	10	25.98		$2X10^6$	$2x10^6$
			There is no significance difference P= 0.01				There is no significance difference P= 0.012		

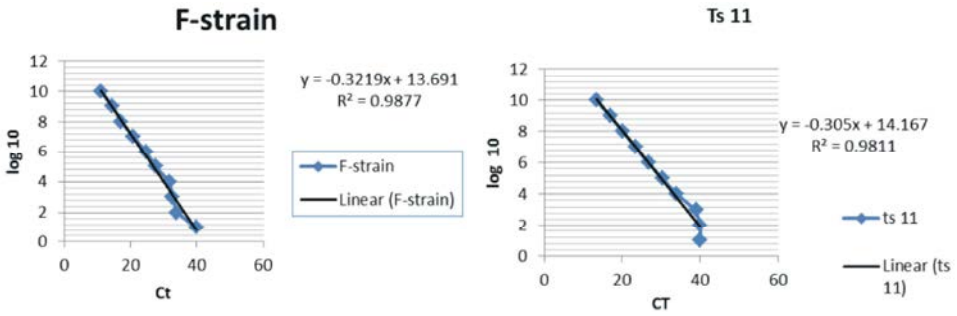

Fig. 3: Standard curve and the calculation equation for F strain (left) and ts-11 MG (right) live vaccine

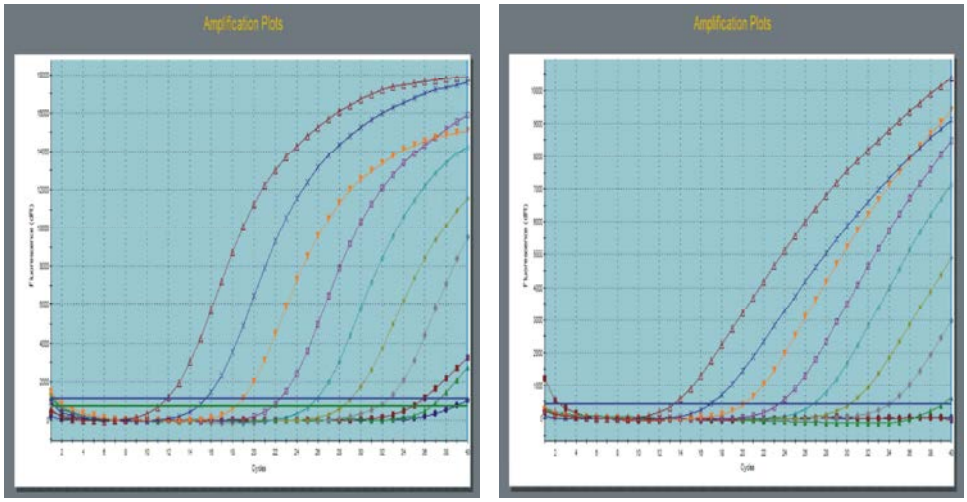

Fig. 4: The amplification plots of rt PCR for F-strain live MG vaccine (left) and ts11 live MG vaccine (right)

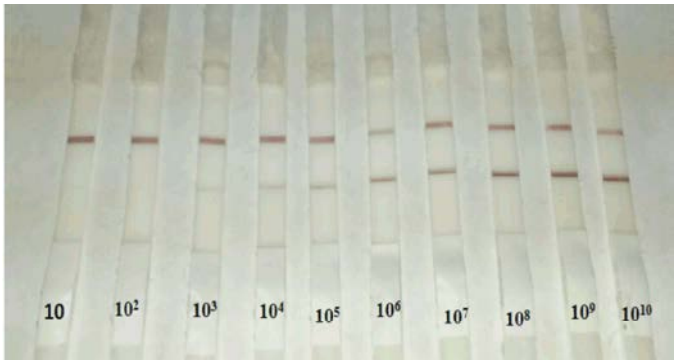

Fig. 5: The sensitivity test of prepared semi-quantitive LFC test for live MG vaccine

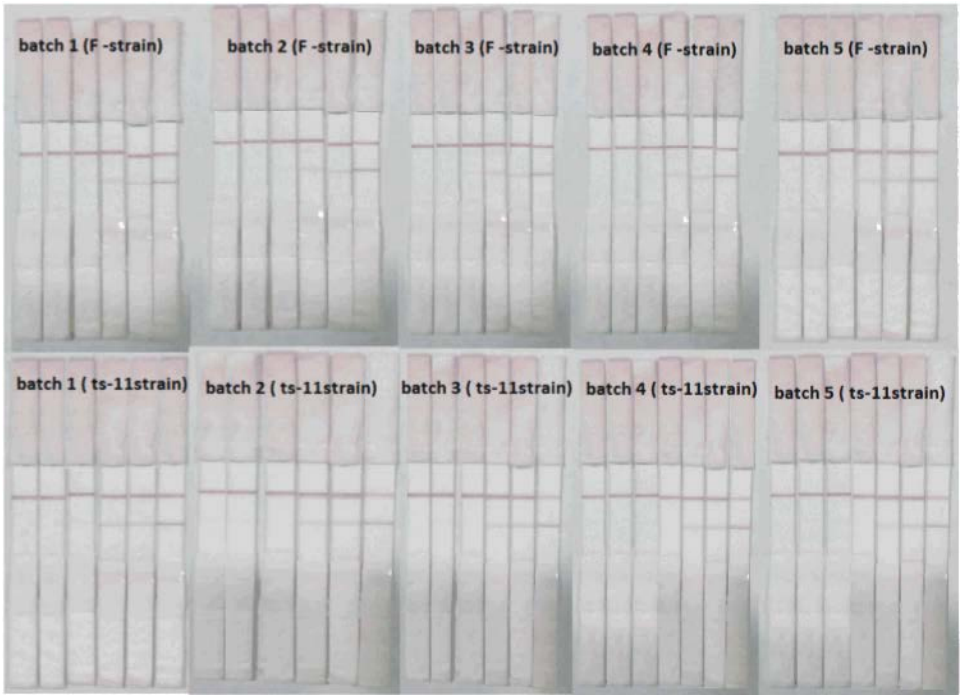

Fig. 6: Shown the semi-quantitive LFC test for of F- strain and ts-11 live MG vaccine

DISCUSSION

The evaluation of live avian mycoplasma vaccines by using traditional method such as total bacterial count (CFU) and colony changing unit (CCU) need a time and specific media for titration in additional to impossible vaccinal strain identification. In this study the two mechanisms were developed rt-PCR and LFC.

The rt-PCR had ability to identify and quantify the two types of vaccines (F-strain and ts-11) strains at the same reaction by using different labeled probe (FAM and HEX) respectively. The sensitivity (minimal CFU that gave positive results) of the rt PCR for F-strain and ts-11 were 10 and 10^3 CFU / sample respectively (Table 2). Ehtisham *et al*. [23] detected 10^2CFU MG / sample using rt-PCR taqman labeled probe while15detected $6.5x10^1$ CFU MG / sample.

The standard curve and linear equation were established to determine the unknown CFU for the new entry vaccine batch within 2 hrs.

Table (3) demonstrated the comparison between rt-PCR titer and bacteriological count for live MG batches 10 batches for each types F-strain and Ts-11 vaccines. The *P*= 0.02 (0.01-0.05) value was 0.01 and 0.012 for F-strain and Ts-11 respectively that's no different significant between the two test.

The minimal CFU MG that give positive for the prepared LFC test was 10^3 CFU / sample, While Chirathaworn *et al*. [13] demonstrated a sensitivity 10 CFU /ml of leptospires using lateral flow devices. Blaskoza *et al*. [24] estimated a sensitivity of 10 CFU/ 25 µl of *Listeria monocytogenes*in dairy productsusing the lateral flow Devices. On the other hand, Wiriyachaiporn *et al*. [25] showed that the lateral flow immunochromatographic devices sensitivity for *S. aureus form* bronchoalveolar lavage samples was 10^6 CFU/ml, while, Yang *et al*. [26] reported that the sensitivity of lateral flow Devices for *E. coli* O157: H7 in bovine feces was 10^5 CFU/ gm.

The LFC test had ability to semi- quantitative the CFU of unknown MG live vaccine within 5 mins Unfortunately the LFC test did not have ability to differentiate between the types of MG vaccine live strain.

We can depend on the LFC test that had ability to determine roughly the count of MG live vaccine with 5min without type identification. On the other hand rt-PCR test had ability to titrate and identify the types of live MG vaccine (F-strain &ts-11) as on reaction for 2hrs.

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