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Original Article

Using the quantitative real-time PCR as alternative method for evaluation of live bacterial poultry vaccines

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Traditional potency evaluation of live bacterial poultry vaccines relies on animal challenge tests, which are labor-intensive, ethically concerning, and subject to biological variability. This study assessed the applicability of quantitative real-time PCR as an *in vitro* alternative method for quantifying antigen content of live bacterial poultry vaccines, and its correlation to protective efficacy. Three commercial live attenuated vaccines (*Mycoplasma synoviae*, *Salmonella* Enteritidis, and *Salmonella* Typhimurium*)* were examined across three independent production batches. Antigen loads were quantified using conventional culture methods (color change units or colony forming units) and quantitative real-time PCR targeting species-specific genes. Five experimental doses, ranging from 2 log units above to 2 log units below the recommended vaccinal dose, were administered to specific pathogen-free chickens followed by homologous challenge. Quantitative real-time PCR assays demonstrated excellent analytical performance, including high linearity ($R^2 \geq 0.998$), acceptable amplification efficiencies, and close agreement with culture-based quantification across all vaccines and batches. Clear dose-dependent protection was observed, with higher quantitative real-time PCR genome copy numbers consistently associated with increased protection rates and a significant positive correlation between antigen loads and protection percentages. In conclusion, quantitative real-time PCR provides a rapid, sensitive, and reproducible method for quantifying live bacterial poultry vaccines, demonstrating a strong correlation with protective efficacy and offering a promising alternative or complementary approach to *in vivo* challenge assays aligning with the principles of animal use reduction.

Keywords: bacterial vaccines; poultry; real-time polymerase chain reaction; microbial colony count; vaccine efficacy.

Introduction

Bacterial diseases remain a major challenge to the poultry industry, causing significant economic losses worldwide. Mycoplasmosis is a key respiratory infection affecting poultry, with *Mycoplasma gallisepticum* and *Mycoplasma synoviae* being the primary pathogenic species.(1)

In addition, enteric bacterial diseases, especially salmonellosis, pose a significant risk to both, poultry production and public health. *Salmonella* Enteritidis and *Salmonella* Typhimurium are prevalent serotypes associated with foodborne infections in humans, mainly through poultry products contamination.(2,3)

Vaccination plays a crucial role in poultry biosecurity programs, particularly in controlling *Mycoplasma* and *Salmonella* infections.(4,5) Traditionally, the potency of live bacterial vaccines has been evaluated through *in vivo* challenge tests, in which vaccinated birds are exposed to the homologous pathogenic strain. Although effective, these tests are labor-intensive, ethically challenging, and subject to biological variability, making standardization and routine batch testing

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complicated. Growing international focus on the application of the 3Rs principles (Replacement, Reduction, and Refinement) has intensified the development of alternative *in vitro* potency assays. For live bacterial vaccines, *in vitro* methods quantifying antigen content have progressively replaced challenge-based assays and provided a clear relationship with protective efficacy.[6] Quantitative real-time PCR (rt-qPCR) is a precise molecular tool for accurately quantifying bacterial genome copies in vaccine formulations.[7] Establishing a quantitative correlation between rt-qPCR derived antigen loads and protection in vaccinated birds could enable the development of a reliable analytical potency assay, reducing or potentially replacing routine animal challenge tests. Therefore, the present study aimed to assess the applicability of using rt-qPCR as an alternative method for quantifying antigen content and evaluating the potency of live *M. synoviae*, *Salmonella* Enteritidis, and *Salmonella* Typhimurium poultry vaccines. It also seeks to investigate the correlation between rt-qPCR results and protection efficacy in vaccinated chickens.

Materials and Methods

Vaccines

Three commercial live attenuated bacterial poultry vaccines were evaluated: *Salmonella* Enteritidis *(*AviPro® SALMONELLA VAC E*), Salmonella* Typhimurium (Aro A gene deleted ST, STM-1 strain), and *M. synoviae* (Vaxsafe® MS ts-11 strain). Three separate commercial batches for each (A, B, and C) were obtained from the same manufacturer.

Bacterial strains for challenge

Virulent strains of *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *M. synoviae* were obtained from the Reference Strain Bank at Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), Abbassia, Cairo, Egypt. These strains were used for homologous challenge experiments.

Experimental animals and housing

A total of 480 one day old specific pathogen free (SPF) chicks were obtained from the SPF egg production farm (Kom Oshim, El-Fayoum, Egypt). Birds were housed under strict biosecurity conditions in HEPA-filtered isolators at the CLEVB animal facility, with controlled temperature, ventilation, and access to feed and water supply.

Vaccine preparation and viable count determination

All vaccine vials were reconstituted according to the manufacturers' instructions. The initial viable antigen content of each batch was determined prior to dose preparation.

Quantification of *Salmonella* vaccines

The viable count of *Salmonella* Enteritidis and *Salmonella* Typhimurium vaccines was determined using the standard surface spread plate method.[6] Ten-fold serial dilutions were prepared in maximum recovery diluent (MRD), plated onto tryptic soy agar (TSA), and then incubated at 37 °C for 24 h. Colonies were enumerated and expressed as colony forming units (CFU) per dose.

Quantification of *Mycoplasma synoviae* vaccine

The viable content of the *M. synoviae* vaccine was determined using the color changing unit (CCU) method.[8] Serial dilutions were prepared in Mycoplasma broth and incubated at 37 °C for up to 14 days. The highest dilution showing a color change from red to yellow was recorded, and CCU per dose was calculated.

Experimental doses preparation

Based on the initial viable count results, five experimental doses were prepared for each vaccine batch: 2 log units above, 1 log unit above, the recommended vaccinal dose, 1 log unit below, and 2 log units below the vaccinal dose. Dose adjustments were achieved through precise dilution in sterile MRD.

Experimental design

A total of 480 chicks were divided into three main groups; *M. synoviae* vaccinated group, *Salmonella* Enteritidis vaccinated group, and *Salmonella* Typhimurium vaccinated group, 160 SPF chicks were

used for each vaccine. Within each main group, 50 chicks were used for each vaccine batch and 10 chicks served as control unvaccinated group. 50 chicks randomly divided into five dose groups with 10 chicks per dose level, each received a different antigen dose (2 log units above, 1 log unit above, the recommended vaccinal dose, 1 log unit below, and 2 log units below the vaccinal dose).

Vaccine inoculation and challenge protocol

Salmonella Enteritidis and *Salmonella* Typhimurium vaccines were orally administered to one-day old chicks. Four weeks post-vaccination, the birds were orally challenged with virulent *Salmonella* Enteritidis ($1x10^7$ CFU) or *Salmonella* Typhimurium ($1x10^6$ CFU). *M. synoviae* vaccine was administered via eye drop at 3 weeks of age, followed by challenge through the abdominal air sac with 1×10^6 CCU at 3 weeks post-vaccination. Birds were monitored for 14 days post-challenge. Protection was evaluated based on clinical signs, mortality, re-isolation, bacterial shedding for *Salmonella* vaccines, and air sac lesion scoring for *M. synoviae* vaccine.

DNA extraction and real-time quantitative PCR

Genomic DNA was extracted from all experimental doses for vaccines evaluation and from high dose for standard curve construction using the Easy Pure® Bacteria Genomic DNA Kit (Trans, Cat. No. EE161) following the manufacturer's instructions.

Standard curves preparation

Ten-fold serial dilutions of extracted DNA were prepared based on known CCU or CFU values per dose. These dilutions were used to generate standard curves for absolute quantification in rt-qPCR assays.

Primers, probes, and rt-quantitative PCR conditions

Species-specific primers and hydrolysis probes targeting the *16S-23S ISR* region for *M. synoviae* and *invA* gene for *Salmonella* spp., were used (Table 1). Reactions were conducted in a 20 µL volume comprising 10 µL of 2× panprobes ™ universal qPCR master mix (QpD01-0100), 0.5 µM of each primer, 0.4 µM of probe, 5 µL of template DNA and nuclease-free water. Amplification was performed on a BIORAD real-time thermocycler under optimized cycling conditions. 95 °C for 10 min followed by 45 cycles at 95 °C for 15 sec, 60 °C for 30 sec for *Mycoplasma* vaccine,[9] and at 94 °C for 15 min followed by 40 cycles at 94 °C for 10 sec and 60 °C for 20 sec for *Salmonella* vaccines.[10]

Correlation and statistical analysis

Cycle threshold (Ct) values and calculated $\log_{10}$ genome equivalents were correlated with protection percentages obtained from challenge studies to determine the antigen thresholds associated with acceptable protection. The correlation coefficient was calculated using SPSS program version 21 (2012) to evaluate the correlation between $\log_{10}$ genome equivalents and protection percentage.

Table 1. Rt-qPCR primers and probes of different vaccines strains.

Vaccine	Primers and probes	Target genes	Reference
M. synoviae	F 5'-CCT-CCT-TTC-TTA-CGG-AGT-ACA-3' R 5'-CTA-AAT-ACA-ATA-GCC-CAA-GGC-AA-3' Probe 5'-FAM-ATT-CTA-AAA-GCG-GTT-GTG-TAT-CGC-T-BHQ1-3	*16s-23S ISR*	WOAH[9]
Salmonella	Sal-F GCGTTCTGAACCTTTGGTAATAA Sal-R CGTTCGGGCAATTCGTTA Sal-Probe FAM-TGGCGGTGGGTTTTGTTGTCTTCT-BHQ1	*invA*	Ibrahim et al.[10]

Ethical approval

All animal procedures were approved by the Institutional Animal Care and Use Committee (ARC-IACUC) of the Agricultural Research Center, under the approval code ARC CLEVB 3-26.

Results

Viable count quantification of vaccine batches

The initial viable antigen concentrations for the three independent batches of *M. synoviae* vaccine were 4.5×10^6, 6.6×10^6, and 5.4×10^6 CCU/dose, while for *Salmonella* Enteritidis were 2.9×10^8, 3.1×10^8, 3.35×10^8 and 4×10^8, 4.5×10^8, and 4.3×10^8 CFU/dose for *Salmonella* Typhimurium vaccine. All vaccine batches met the expected viable count ranges and were then used to prepare experimental doses 2 log and 1 log units above and below the recommended vaccinal dose.

Standard curve performance

The analytical performance of standard curves for three vaccines (Table 2), exhibited excellent linearity across the tested concentration ranges, with coefficient of determination (R^2) ranging from 0.9986- 0.9999 and amplification efficiencies ranging from 99.5 % to 104 %. The regression equations yielded consistent slopes (approximately -3.23 to -3.29). These findings confirm the robustness, sensitivity, and reproducibility of the developed rt-PCR assays for quantitative evaluation of the three live bacterial vaccines.

Rt-qPCR quantification of experimental vaccine doses

Rt-qPCR assays were successfully developed in all three live attenuated bacterial poultry vaccines using DNA extracted from three independent production batches (Tables 3). Genome equivalent values for the five graded experimental doses closely paralleled the corresponding viable counts obtained by conventional culture methods across all three independent batches. A consistent logarithmic reduction in Ct values of approximately 3.2 - 3.4 cycles per $\log_{10}$ dilution was observed with increasing antigen concentration, confirming the expected exhibited inverse linear relationship between Ct values and template quantity. Across the tested dose ranges, rt-qPCR quantification demonstrated strong agreement with culture-based enumeration, with only minor numerical variations that remained within acceptable analytical limits. Minimal inter-batch variability was observed, indicating high production consistency and assay repeatability.

Table 2. Standard curve parameters of rt-qPCR assays.

M. synoviae		***Salmonella* Enteritidis**		***Salmonella* Typhimurium**	
Standard curve	**Ct values**	**Standard curve**	**Ct values**	**Standard curve**	**Ct values**
10^8	13.43	10^{10}	15.2	10^{10}	15.5
10^7	16.71	10^9	18.4	10^9	18.6
10^6	19.60	10^8	21.7	10^8	21.8
10^5	22.88	10^7	25.1	10^7	25.1
10^4	26.51	10^6	28.3	10^6	28.5
Linear equation Y= -3.233X+39.224		**Linear equation** Y= -3.29X+48.06		**Linear equation** y = -3.25x + 47.9	
R squared 0.9986		**R squared** 0.9999		**R squared** 0.9997	
Amplification efficiency (%) 101.5%		**Amplification efficiency (%)** 99.5%		**Amplification efficiency (%)** 104%	

Ct: cycle threshold.

Table 3. Absolute quantification of five experimental doses of *M. synoviae*, *Salmonella* Enteritidis, and *Salmonella* Typhimurium vaccines using rt-qPCR.

Batch	*M. synoviae*			*Salmonella* Enteritidis			*Salmonella* Typhimurium		
	Viable count (experimental doses)	rt -qPCR	Ct values	Viable count (experimental doses)	rt -qPCR	Ct values	Viable count (experimental doses)	rt -qPCR	Ct values
A	4.5×10^{8}	5×10^{8}	11.1	2.9×10^{10}	3.5×10^{10}	13.4	4×10^{10}	4.4×10^{10}	13.3
	4.5×10^{7}	4.9×10^{7}	14.4	2.9×10^{9}	4×10^{9}	16.5	4×10^{9}	4.8×10^{9}	16.4
	4.5×10^{6}	5.1×10^{6}	17.6	2.9×10^{8}	3×10^{8}	20.2	4×10^{8}	4×10^{8}	20
	4.5×10^{5}	5.4×10^{5}	20.8	2.9×10^{7}	3.9×10^{7}	23.1	4×10^{7}	5×10^{7}	22.9
	4.5×10^{4}	5×10^{4}	24	2.9×10^{6}	4.2×10^{6}	26.3	4×10^{6}	5.4×10^{6}	26.1
B	6.6×10^{8}	7×10^{8}	10.6	3.1×10^{10}	4.5×10^{10}	13.02	4.5×10^{10}	5.4×10^{10}	13.03
	6.6×10^{7}	7.5×10^{7}	13.7	3.1×10^{9}	4.8×10^{9}	16.2	4.5×10^{9}	4.8×10^{9}	16.4
	6.6×10^{6}	6.8×10^{6}	17.1	3.1×10^{8}	4×10^{8}	19.8	4.5×10^{8}	5×10^{8}	19.6
	6.6×10^{5}	8×10^{5}	20.1	3.1×10^{7}	3.5×10^{7}	23.3	4.5×10^{7}	5×10^{7}	22.9
	6.6×10^{4}	8.4×10^{4}	23.3	3.1×10^{6}	5×10^{6}	26.02	4.5×10^{6}	5.6×10^{6}	26
C	5.4×10^{8}	7×10^{8}	10.6	3.35×10^{10}	4×10^{10}	13.2	4.3×10^{10}	6×10^{10}	12.9
	5.4×10^{7}	6.8×10^{7}	13.9	3.35×10^{9}	5×10^{9}	16.1	4.3×10^{9}	5.8×10^{9}	16.2
	5.4×10^{6}	8.4×10^{6}	16.9	3.35×10^{8}	3.7×10^{8}	19.8	4.3×10^{8}	5.4×10^{8}	19.5
	5.4×10^{5}	6×10^{5}	20.6	3.35×10^{7}	5.3×10^{7}	22.7	4.3×10^{7}	4.9×10^{7}	22.9
	5.4×10^{4}	7.6×10^{4}	23.4	3.35×10^{6}	4.6×10^{6}	26.1	4.3×10^{6}	5×10^{6}	26.1

Ct: cycle threshold

Dose dependent protective efficacy

All three live bacterial vaccines elicited clear dose-dependent protective response following homologous challenge (Table 4). In case of *M. synoviae,* higher experimental doses achieved protection rates of approximately 76 - 78 %. In contrast, lower antigen doses resulted in a gradual decline in protection level. The protection level observed for *Salmonella* Enteritidis and *Salmonella* Typhimurium exceeded 80 % at the highest antigen doses and remained at approximately 74 -78 % at the recommended vaccinal dose. Then declined to 70 % at the lower doses. Unvaccinated control groups showed low protection levels (18 - 20 %), confirming the validity of the challenge models. Protection trends were consistent across all vaccine batches.

Table 4. Dose-dependent protective efficacy of live bacterial poultry vaccines following homologous challenge.

Vaccine batch	*M. synoviae*		*Salmonella* Enteritidis		*Salmonella* Typhimurium	
	Experimental and vaccinal doses	Protection %	Experimental and vaccinal doses	Protection %	Experimental and vaccinal doses	Protection %
A	4.5×10^{8}	77 %	2.9×10^{10}	83 %	4×10^{10}	84 %
	4.5×10^{7}	75 %	2.9×10^{9}	81 %	4×10^{9}	80 %
	4.5×10^{6}	72 %	2.9×10^{8}	76 %	4×10^{8}	78 %
	4.5×10^{5}	70 %	2.9×10^{7}	75 %	4×10^{7}	74 %
	4.5×10^{4}	68 %	2.9×10^{6}	70 %	4×10^{6}	70 %
B	6.6×10^{8}	76 %	3.1×10^{10}	79 %	4.5×10^{10}	78 %
	6.6×10^{7}	74 %	3.1×10^{9}	77 %	4.5×10^{9}	76 %
	6.6×10^{6}	73 %	3.1×10^{8}	75 %	4.5×10^{8}	74 %
	6.6×10^{5}	70 %	3.1×10^{7}	72 %	4.5×10^{7}	72 %
	6.6×10^{4}	65 %	3.1×10^{6}	70 %	4.5×10^{6}	70 %
C	5.4×10^{8}	78 %	3.35×10^{10}	83 %	4.3×10^{10}	82 %
	5.4×10^{7}	76 %	3.35×10^{9}	81 %	4.3×10^{9}	80 %
	5.4×10^{6}	72 %	3.35×10^{8}	76 %	4.3×10^{8}	75 %
	5.4×10^{5}	70 %	3.35×10^{7}	75 %	4.3×10^{7}	72 %
	5.4×10^{4}	66 %	3.35×10^{6}	70 %	4.3×10^{6}	70 %
	Control	18 %	**Control**	20 %	**Control**	20 %

Table 5. Correlation between rt-qPCR antigen quantification and protection percentage of live bacterial poultry vaccines across vaccine batches.

Vaccine batch	*M. synoviae*		*Salmonella* Enteritidis		*Salmonella* Typhimurium	
	rt-qPCR quantification of experimental doses	Protection %	rt-qPCR quantification of experimental doses	Protection %	rt-qPCR quantification of experimental doses	Protection %
A	5×10^{8}	77 %	3.5×10^{10}	83 %	4.4×10^{10}	84 %
	4.9×10^{7}	75 %	4×10^{9}	81 %	4.8×10^{9}	80 %
	5.1×10^{6}	72 %	3×10^{8}	76 %	4×10^{8}	78 %
	5.4×10^{5}	70 %	3.9×10^{7}	75 %	5×10^{7}	74 %
	5×10^{4}	68 %	4.2×10^{6}	70 %	5.4×10^{6}	70 %
B	7×10^{8}	76 %	4.5×10^{10}	79 %	5.4×10^{10}	78 %
	7.5×10^{7}	74 %	4.8×10^{9}	77 %	4.8×10^{9}	76 %
	6.8×10^{6}	73 %	4×10^{8}	75 %	5×10^{8}	74 %
	8×10^{5}	70 %	3.5×10^{7}	72 %	5×10^{7}	72 %
	8.4×10^{4}	65 %	5×10^{6}	70 %	5.6×10^{6}	70 %
C	7×10^{8}	78 %	4×10^{10}	83 %	6×10^{10}	82 %
	6.8×10^{7}	76 %	5×10^{9}	81 %	5.8×10^{9}	80 %
	8.4×10^{6}	72 %	3.7×10^{8}	76 %	5.4×10^{8}	75 %
	6×10^{5}	70 %	5.3×10^{7}	75 %	4.9×10^{7}	72 %
	7.6×10^{4}	66 %	4.6×10^{6}	70 %	5×10^{6}	70 %
	Control	18 %	**Control**	20 %	**Control**	20 %

Ct: cycle threshold.

Correlation between rt-qPCR antigen load and protection percentage

A strong positive correlation was observed between rt-qPCR-derived antigen loads and protection percentages across all vaccines and production batches (Table 5). Increasing genome equivalent values were consistently associated with higher protection rates, whereas lower rt -qPCR values corresponding to reduced protection. Correlation coefficients ranged from 0.958 to 0.997 across all batches and vaccines and were statistically significant ($p< 0.05$). These results demonstrating that rt -qPCR-based antigen quantification reliably predicts biological protective efficacy.

Discussion

Accurate quantification of live bacterial poultry vaccines is essential for ensuring batch consistency and protective efficacy. In this study, conventional culture-based methods (CFU for *Salmonella* vaccines and CCU for *M. synoviae* vaccine) were successfully applied to quantify the initial viable content of three independent production batches, demonstrating acceptable batch-to-batch uniformity providing a reliable basis for graded experimental doses preparation. These findings align with previous reports supporting viable counts as potency indicators for live bacterial poultry vaccines.[6]

Although culture-based assays remain a reference standard, they are time-consuming and prone to variability related to growth characteristics, incubation conditions, and operator handling, especially for fastidious organisms such as mycoplasmas. In this context, molecular methods offer a practical advancement by integrating rapid quantification with biological efficacy data. In the present study, rt-qPCR assays exhibited excellent analytical performance, with high linearity ($R^2 \geq 0.998$), optimal amplification efficiencies, consistent Ct shifts across serial dilutions, and strong concordance with culture-based counts. This agreement supports rt-qPCR as a surrogate indicator of antigen load in live bacterial vaccines. In general, rt-qPCR yielded slightly higher values than culture-based quantification, reflecting detection of total bacterial DNA, including viable and non-viable cells. Several findings have been reported for *Salmonella* and *Mycoplasma* vaccines, where real-time PCR assays were shown to be rapid, sensitive, and reliable alternatives to conventional culture techniques.[10,11,12,13,14]

Protection studies revealed a clear and reproducible dose -response relationship across all evaluated vaccines and batches, with higher antigen doses yielded greater protection, while lower doses resulted in reduced efficacy. For *M. synoviae*, protection was manifested primarily as reduced air sac lesions and disease severity. Complete sterile immunity was not observed, which is consistent with the known biological behavior of mycoplasma infections. For *Salmonella* Enteritidis and *Salmonella* Typhimurium, reduced antigen doses were associated with reduction in clinical signs, lesion severity, increased bacterial recovery and shedding following homologous challenge aligning with recommendations[9] for evaluation of live *Salmonella* vaccines in poultry. The marked decrease in protection at lower antigen doses further demonstrates a clear dose response relationship, as required for potency assessment. The low protection levels observed in unvaccinated control groups confirm the adequacy and validity of the challenge models, fulfilling criteria[9] for challenge study design. Additionally, the consistent protection trends observed across all vaccine batches comply with European Pharmacopoeia requirements[15] for batch - to batch consistency and reproducibility of vaccine potency for live bacterial veterinary vaccines.

A key objective of this study was to evaluate the relationship between rt-qPCR-derived antigen quantification and *in vivo* protective efficacy. Significant positive correlation between rt-qPCR genome equivalents and protection percentages was demonstrated across all vaccines and batches. Although rt-qPCR quantifies total bacterial DNA including non-viable organisms, the strong association with *in vivo* efficacy suggests that total antigenic mass is a key driver of immune stimulation in live attenuated vaccines, even with partial viability loss during production or storage. These findings indicate that molecularly quantified antigen load is predictive of biological vaccine performance and supports the use of rt-qPCR as a potency-related analytical assay.

Based on combined molecular quantification and protection data, minimum release doses were established for each vaccine, corresponding to the lowest antigen concentration that consistently achieved acceptable protection. For *M. synoviae* vaccine, approximately 5×10^5 or more antigen concentrations were associated with protection levels 70 %, while *Salmonella* Enteritidis and *Salmonella* Typhimurium vaccines required minimum antigen loads of approximately 4-5×10^6 to achieve comparable protection. Higher doses, 1 log and 2 log units above the recommended vaccinal dose showed no adverse clinical effects for *Salmonella* vaccines, indicating a substantial safety margin and supporting the biological tolerability of higher antigen loads.[6] For *M. synoviae* vaccine, higher doses, 1 log and 2 log units above the recommended vaccinal dose produced mild air sac lesions, but not sever enough to be classified as grade one on the lesion scale so, the maximum release dose was considered 10^7 CCU/dose.

From a regulatory perspective, international guidelines[6] emphasize the development of alternative potency assays justified by correlation with *in vivo* protection. The strong correlation observed between rt-qPCR results and protective efficacy in the present study fulfills this requirement and supports the potential implementation of rt-qPCR as an alternative or complementary assay for routine batch release testing of live bacterial poultry vaccines. Further validation including additional live bacterial poultry vaccines such as *M. gallisepticum* and *E. coli* would further broaden the applicability of this approach.

Conclusions

This study demonstrates that rt-qPCR is a rapid, sensitive, and reproducible method for quantifying antigen content in live bacterial poultry vaccines. The strong positive correlation observed between rt-qPCR-derived genome equivalents and protection efficacy confirm the biological relevance of molecular quantification as a reliable potency indicator. The identification of minimum protective antigen threshold, combines with the confirmed safety of higher antigen doses, supports the potential implementation of rt-qPCR as an alternative or complementary assay to *in vivo* challenge tests for routine batch release. Adoption of such molecular approaches could substantially reduce animal use while maintaining robust assurance of vaccine quality and efficacy.

Conflict of interest

The authors declare that there is no conflict of interest.

Author´s contributions

Afaf A. Khedr: designed and followed up the experiment and critically reviewed the manuscript, participated in designing and followed up the practical work.

Fatma El-Zahraa Gamal: conducted the experiment and drafted the manuscript, designed and followed up the experiment and critically reviewed the manuscript.

Eman Soliman: designed and followed up the experiment and critically reviewed the manuscript.

Samir A. Nassif: conducted the experiments and drafted the manuscript, designed and followed up the experiment and critically reviewed the manuscript.

All authors have read and agreed to the published version of the manuscript.

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Utilización de la PCR cuantitativa en tiempo real como método alternativo para la evaluación de vacunas bacterianas vivas para aves de corral

Resumen

La evaluación tradicional de la potencia de las vacunas bacterianas vivas para aves de corral se basa en pruebas de desafío en animales, las cuales son laboriosas, plantean problemas éticos y están sujetas a variabilidad biológica. Este estudio evaluó la aplicación de la PCR cuantitativa en tiempo real como método alternativo *in vitro* para cuantificar el contenido antigénico de las vacunas bacterianas vivas para aves de corral y su correlación con la eficacia protectora. Se analizaron tres vacunas vivas atenuadas comerciales (*Mycoplasma synoviae*, *Salmonella* Enteritidis y *Salmonella* Typhimurium) en tres lotes de producción independientes. Las cargas antigénicas se cuantificaron mediante métodos de cultivo convencionales (unidades de cambio de color o unidades formadoras de colonias) y PCR cuantitativa en tiempo real dirigida a genes específicos de cada especie. Se administraron cinco dosis experimentales, que oscilaron entre 2 unidades logarítmicas por encima y 2 unidades logarítmicas por debajo de la dosis vacunal recomendada, a pollos libres de patógenos específicos, seguidas de un desafío homólogo. Los ensayos de PCR cuantitativa en tiempo real demostraron un excelente rendimiento analítico, incluyendo una alta linealidad ($R^2 \geq 0,998$), eficiencias de amplificación aceptables y una estrecha concordancia con la cuantificación basada en cultivos en todas las vacunas y lotes. Se observó una clara protección dependiente de la dosis, con un mayor número de copias del genoma en la PCR cuantitativa en tiempo real asociado consistentemente con mayores tasas de protección y una correlación positiva significativa entre las cargas antigénicas y los porcentajes de protección. En conclusión, la PCR cuantitativa en tiempo real proporciona un método rápido, sensible y reproducible para cuantificar las vacunas bacterianas vivas para aves de corral, demostrando una fuerte correlación con la eficacia protectora y ofreciendo un enfoque alternativo o complementario prometedor a los ensayos de desafío *in vivo*, en consonancia con los principios de reducción del uso de animales.

Palabras clave: vacunas bacterianas; aves de corral; reacción en cadena en tiempo real de la polimerasa; recuento de colonia microbiana; eficacia de las vacunas.

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