PERFORMANCE VALIDATION OF QUANTITATIVE POLYMERASE CHAIN REACTION ASSAYS FOR MEASURING SWINE PROINFLAMMATORY AND IMMUNOMODULATORY CYTOKINE GENE EXPRESSION

Validación de una PCR cuantitativa para la medición de la expression genética de citokinas proinflamatorias e inmunomoduladoras

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ABSTRACT

RT-qPCR is the method of choice for the accurate detection of low quantities of mRNA due to its higher sensitivity and specificity. Most of the previously published reports about swine cytokine gene expression lack information regarding the validation of the technique, which impedes the potential implementation by new users. This study was focussed on the technical validation of already published RT-qPCR assays for swine proinflammatory (IFN-α, IFN-γ, IL-12p35, IL-12p40 and TNF-α) and immunomodulatory (IL-10 and TGF-β)

cytokines, and on defining the best qPCR amplification conditions for simultaneous amplification of the selected cytokines from several porcine tissue samples. The tested RT-qPCR assays here are sensitive (Efficient close to 2, Correlation coefficient higher than 0.95 and a Limit Of Detection below 305-100 mRNA copies), robust (Coefficint of Variation and Factor of Discrimination means were lower than 5 and 3%, respectively) and highly useful for the study of immune swine responses.

Keywords*:* swine; cytokines; quantitative PCR; validation.

RESUMEN

La alta sensibilidad y especificidad de la RT-qPCR le hacen el método de elección para la detección precisa de bajas cantidades de ARNm. La mayoría de artículos previamente publicados sobre expresión de citocinas porcinas carecen de información acerca de la validación de la técnica empleada, lo que dificulta su aplicación por nuevos usuarios. El presente estudio se centra en la validación de técnicas de RT-qPCR previamente publicadas para citocinas proinflamatorias (IFN-α, IFN-γ, IL-12p35, IL-12p40 y TNF-α) e inmunomoduladoras (IL-10 y TGF-β). Además se definen las mejores condiciones de amplificación simultánea para qPCR de las citadas citocinas en tejidos porcinos. Los ensayos de RT-qPCR evaluados son sensibles (Eficiencia cercana a 2, coeficiente de correlación mayor de 0.95 y un límite de detección entre 305-100 copias de ARNm), son robustas (coeficiente de variación y factor de discriminación menores de 5 y 3%, respectivamente), y don altamente útiles para el estudio de la respuesta inmune en el cerdo.

Palabras clave: cerdo; citocinas; PCR cuantitativa; validación.

INTRODUCTION

Studies of the immune system by analyzing cytokine expression provide a deep insight into disease pathogenesis. Changes in the cytokine production during infection may be used as markers of both the protective immunity and the outcome of the disease (Corradi *et al*., 2007; Gómez-Laguna *et al*., 2013). The measurement of cytokine messenger ribonucleic acid (mRNA) represents an alternative to immunoassays for the detection of cytokines that sometimes are difficult to detect due to their low quantities (Quereda *et al*., 2013). Furthermore, the detection of cytokine mRNA provides a more sensitive detection method than immunoassays for pathological damage associated to infectious agents (Whiteside, 1994). However, variations in mRNA induction may occur at transcriptional level, being required a complementary study among techniques to evaluate in depth the immune response (Verfaille *et al*., 2001).

An important application of reverse transcription quantitative polymerase chain reaction (RT-qPCR) is to measure the antigen specific immune response of small populations of cells against pathogens (Coussens *et al*., 2006). The measurement of gene expression has undergone a revolution in the last decade with the emergence of quantitative polymerase chain reaction (qPCR). RT-qPCR is established as the current method of choice for the accurate detection of low quantities of mRNA due to its higher sensitivity and specificity than other laboratory techniques (Segalés *et al*., 2005; Bustin *et al*., 2010). For research applications, a two-step protocol for RT-qPCR provides more flexibility, sensitivity and potential for optimization (Nolan *et al*., 2009). This technique has allowed accurate quantification of mediators of the pig immune response against infection and/or vaccination, allowing the profiling of the transcriptional states of cells and tissues (Kasprovicz *et al*., 2011).

Two of the main obstacles impeding a more extensive adoption of RT-qPCR assays for clinical use are concerns over assay quality assessment and standardization, both of which affect reproducibility (Murphy and Bustin, 2009). qPCR efficiency could be affected among others by the amplicon size, primer length, annealing

temperature and secondary structures (Bustin and Nolan, 2004). Most of the reports previously published about swine cytokine gene expression lack information regarding reproducibility, efficiency and specificity of the assays (Bustin *et al*., 2009). Due to this limitation, it remains difficult for new researchers to evaluate the utility of these assays for quantification of cytokine gene expression in their specific experimental conditions (e.g. induction of the gene under study).

The purpose of the present study was to validate the performance of published RT-qPCR assays for several swine proinflammatory and immunomodulatory cytokines. The main intention was to evaluate its potential application as sensitive and reliable assays on porcine tissues samples. The secondary aim was to define the best qPCR amplification conditions that allow the simultaneous amplification of all of these cytokines to provide a fast tool to monitor the proinflammatory and immunomodulatory pig responses after naturally occurring or experimental infections. Primers for each proinflammatory [Interferon α (IFN-α), Interferon γ (IFN-γ), Interleukin 12p35 (IL-12p35), IL-12p40 and Tumor Necrosis Factor α (TNF-α)] and immunomodulatory [IL-10 and Transforming Growth Factor β (TGF-β)] cytokine were selected from previously published reports (integrated into the Porcine Immunology Nutrition Database, http://www.ars.usda.gov/services/docs.htm?docid=6065), which lack information regarding reproducibility and efficiency of the assays (Royaee *et al*., 2004; Gabler *et al*., 2006; Moue *et al*., 2007; Kim *et al*., 2010).

MATERIAL AND METHODS

mRNA extraction

To carry out this study a pool of retropharyngeal lymph nodes, tonsil and lung samples collected from three landrace x large white pigs, experimentally infected with a type 1 porcine reproductive and respiratory syndrome virus (PRRSV) strain and killed at 3, 10 and 21 days post infection were obtained from previous experiments of our group based on an enhanced protein expression of the selected cytokines (Gómez-Laguna *et al*., 2010; Gómez-Laguna *et al*., 2012; Barranco *et al*., 2012ab). Tissue samples were immediately frozen in liquid nitrogen within 1 min after euthanization, and stored at -80°C until laboratory processing. The samples were dissected by forceps to obtain 20 mg of tissue and a pool of tissues were done in order to extract RNA with the RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA) following the guidelines of the manufacturer. RNA was treated with DNase by using the Turbo DNA-free™ kit (Ambion, Austin, Tx, USA) following the manufacturer's instructions. The concentration and the purity of mRNA and cDNA samples were assessed with the ratio of absorbance at 260/280 and 260/230 nm using a Nanodrop ND 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). RNA integrity was assessed by agarose-TAE electrophoresis and RNA was stored at -80ºC until using.

cDNA synthesis

cDNA was synthesized from total RNA obtained by using OligodT/random hexamers and the GeneAmp RNA PCR Core kit (Applied Biosystems, USA) with MuLV Reverse Transcriptase and RNase Inhibitor in a 20μl volume reaction containing 20 U enzyme, 1 μg total RNA, 100 ng/μl random hexamers, 10mM DNTP mix and DEPC treated water. The reaction was carried out for 15 min at 42ºC followed by inactivation at 99ºC for 5 min. cDNA was diluted at different concentrations (ratio cDNA:water 1:1, 1:5, and 1:25) and stored at -80°C until laboratory processing.

Primers

All primers were synthesized by TIB Molbiol (Berlin, Germany). Table 1 lists the se-

Table 1.

Cytokine	Primer Forward	Primer Reverse	Amplicon Length	Locus	Reference
IFN-α	5'-CCCCTGTGCCTGGGAGAT-3'	5'-AGGTTTCTGGAGGAAGAGAAGGA-3'	**63 bp**	**XM_003480507.1**	16
IFN-γ	5'-TGGTAGCTCTGGGAAACTGAATG-3'	5'-GGCTTTGCGCTGGATCTG-3'	**79 bp**	**NM_213948**	14
TNF-α	5'-ACTCGGAACCTCATGGACAG-3'	5'-AGGGGTGAGTCAGTGTGACC-3'	**134 bp**	**X_54859.1**	15
IL-12p35	5'-AGTTCCAGGCCATGAATGCA-3'	5'-TGGCACAGTCTCACTGTTGA-3'	**84 bp**	**NM_213993.1**	16
IL-12p40	5'-TTTCAGACCCGACGAACTCT-3'	5'-CATTGGGGTACCAGTCCAAC-3'	**160 bp**	**NM_214013.1**	17
IL-10	5'-TGAGAACAGCTGCATCCACTTC-3'	5'-TCTGGTCCTTCGTTTGAAAGAAA-3'	**109 bp**	**NM_214041**	14
TGF-β	5'-CACGTGGAGCTATACCAGAA-3'	5'-TCCGGTGACATCAAAGGACA-3'	**108 bp**	**AF_461808.1**	16

Primers sequences, amplicon size, locus and references of each qPCR assay.

quences of the forward and reverse primers. All primers pair produced amplicons smaller than 160 base pair (bp).

qPCR

Synthesized cDNA was amplified using the ABI 7300 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Absolute quantification real time qPCR was performed using SYBR green chemistry (Power SYBR Green, Applied Biosystems, Foster City, CA, USA) for a total volume of 25 μl. Thermal cycle conditions were 10 minutes at 95ºC, 40 cycles of 95ºC for 15 seconds, and annealing-extension at 60ºC for one minute for all tested primers. Reverse transcription negative controls and non template controls were included. Finally, a dissociation curve was performed with 110 cycles of denaturation at 90ºC for 15 seconds and renaturation at 60ºC for one minute with a temperature decrease of 0.3ºC for each cycle, in order to ensure that a single product had been amplified and that no contamination was present in the reverse transcription negative controls or in the non template controls. All real time PCR reactions were performed in a 96 well reaction plates (Applied Biosystems, Foster City, CA, USA).

qPCR was optimized for target specificity and primer concentration. The qPCRs assays were done combining different cDNA concentration (ratio cDNA:water 1:1, 1:5, and 1:25) and primer concentration for each cytokine assay (50, 200, 300 and 900 nM). All possible combinations of cDNA and primer concentration were run in duplicate. The specificity of amplification was evaluated by gel electrophoresis in 2% (w/v) agarose gel in 1 x TAE (Tris Acetate-EDTA buffer; Sigma-Aldrich, St. Louis, MO, USA), and RedSafe™ (Intron Biotechnology, Seongnam, South Korea) staining. The best qPCR combination of cDNA and primers concentrations was selected for each cytokine depending on two conditions: (i) absence of primer dimers; and, (ii) minimum quantification cycle value (Cq) at same amount of cDNA. Cq is defined as the cycle number at which the SYBR-Green-DNA interaction fluorescent signal attained a threshold level avoiding the background fluorescence (Trichopad *et al*., 2010).

Calculation of qPCR parameters

In order to generate the standard curves the transcript of each cytokine of interest was

encoded in a plasmid (pGEM-T easy plasmid, Promega) following the manufacturer's instructions. The plasmids were cut using restriction enzymes Pst I or Xmn I (Promega), depending on the cytokine nucleotide sequence. The plasmids with the inserts were sequenced using an ABI Prism 3130 (Applied Biosystems, Foster City, CA, USA). Dilutions were prepared in DNase and RNase free MiliQ water such that the final copy number in the qPCR assay ranged from 10^8-10^1 copies/μl for 10-fold dilutions and from 10^7-305 copies/µl for 2-fold dilutions. Replicates for 10-fold and 2-fold serial dilutions were completed in quintuplicate which indicates the repeatability of qPCR assays. No-template controls were included for each standard curve. Negative RT controls were performed to exclude the possibility of genomic contamination. The thermocycler conditions were the same that described above for qPCR.

Analysis of the assay performance included determination of the amplification efficiency, coefficient of variation and factor of discrimination.

Efficiency (E) was calculated with the following formula: $E = 10^{\frac{-1}{slope}}$. The qPCR efficiency percentage was calculated applying the formula $\%E = \frac{E \times 100}{2}$. An E value of 2 implies that two copies of DNA are generated from every template during the amplification phase of PCR reaction in each cycle. The slope was provided by the ABI 7300 Real Time PCR system using the ABI PRISM Sequence Detection Software, version 1.3.1 (Applied Biosystems, Foster City, CA, USA) to calculate the slope.

Coefficient of variation (CV) was calculated with the following formula: $CV = \left(\frac{SD}{Cq\bar{X}}\right) \times 100$; where SD is the standard deviation of replicates of the same amount of template, and Cq average is the arithmetic mean of the Cq values in the replicates of the same amount of template. The CV shows the extent of variability in relation to mean of the Cq.

Factor of Discrimination (FD) was calculated with the following formula: $FD = E^2 \times SD$; where E is the efficiency and SD is the standard deviation of replicates for the same template. The FD determines the assay ability to distinguish different amounts of DNA along the fold dynamic range. This parameter estimates the amplitude between the maximum and minimum Cq values in the replicates of the same amount of cDNA template.

All parameters were calculated using Microsoft Office Excel 2007 (Microsoft Co., Redmond, WA, USA). The analytical sensitivity of these qPCR assays was characterized by the limit of detection (LOD), taken as the last concentration in which all qPCR replicates were detected with a CV% lower than 2%. The detection limits of the assays were assessed using 10-fold and 2-fold dilutions of the plasmids.

RESULTS

mRNA, cDNA and plasmid quality

All the values obtained at 260/280 nm were between 1.8 and 1.9. All the values obtained at 260/230 nm were between 2.0 and 2.2; and the electrophoresis showed good quality RNA bands. cDNA was diluted at previously indicated ratios after the spectrophotometric assay.

Primers specificity and concentration

In order to assess primers specificity, a BLAST in silico tool was used for all primers and resulted in 100% homology to target genes. Primer concentration for each cytokine was selected based on the lowest Cq value obtained with the lowest amount of cDNA. The best primer concentration for each cytokine was: 200 nM for IFN-γ, 300nM for IFN-α, TNF-α, IL-10 and TGF-β, and 900 nM for IL-12p35 and IL-12p40. Specific single bands within the expected amplicon sizes were obtained by 2% agarose gel electrophoresis (Figure 1) which was confirmed by DNA sequencing of the PCR products using an ABI Prism 3130 (Applied

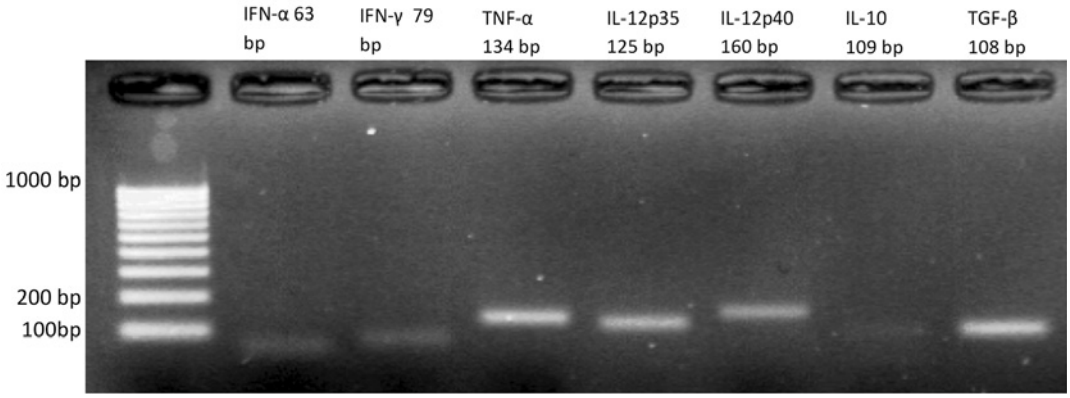


Figure 1. **Agarose gel electrophoresis of the real time qPCR product of each cytokine.**

Biosystems, Foster City, CA, USA). The analysis of the dissociation stage at the end of the qPCR showed dissociation curves with single peaks for IFN-γ, TNF-α and IL-12p35. A small peak preceding the main peak was observed for IFN-α, IL-12p40, IL-10 and TGF-β (Figure 2). Since these small peaks were not identified as PCR products bands in gel electrophoresis, they had smaller intensity signals and they do not appear in no-template controls, we assumed that they were an artefact and do not significantly affect template quantification (as shown by qPCR efficiency). No primer dimer formation was observed in no-template or negative RT controls for any cytokine.

qPCR parameters

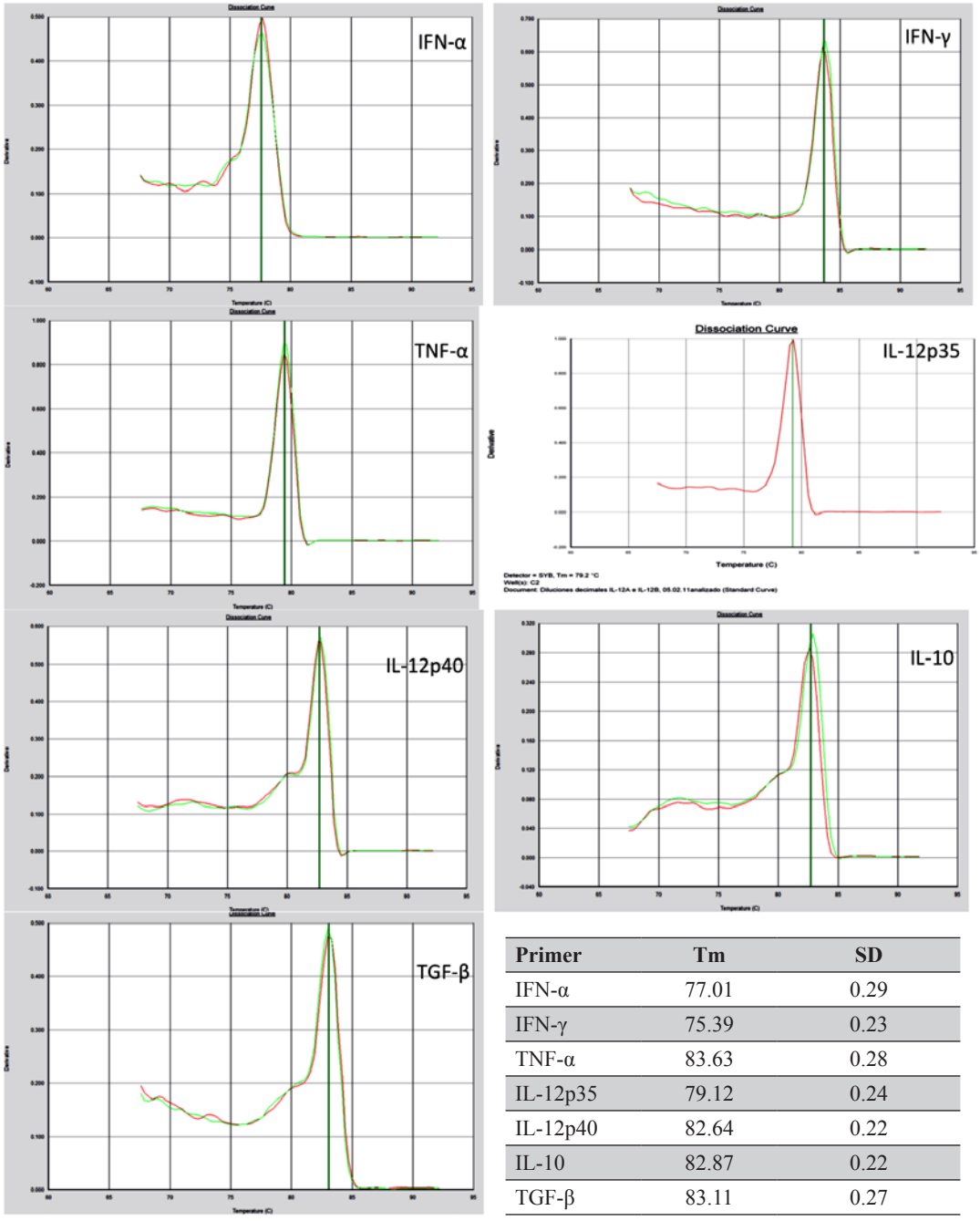
All qPCR amplification plots displayed amplification curves with an exponential phase followed by a non-exponential phase, ending with a plateau. The linear plots of the baseline corrected for 2-fold and 10-fold dilutions qPCR are represented in Figure 3 and Figure 4, respectively.

The LOD was 100 copies for the qPCR assays of IFN-α, IFN-γ, IL-12p35, IL-12p40 and IL-10. In the case of TGF-β and TNF-α qPCR assays, the LOD was 305 copies.

The ABI 7300 software (Applied Biosystems, Foster City, CA, USA) showed for all qPCR assays correlation coefficients of $R^2 > 0.95$ (Table 2) except for IL-10 2-fold dilution ($R^2 = 0.75$). E values ranged between 1.68 (TNF-α) and 2.38 (IL-10) using a plasmid DNA template (Table 2). The E and percentage of E of each 2-fold and 10-fold serial dilution qPCR for each cytokine are summarized in Table 2.

The CVs for each cytokine are represented in Table3. The CV in the 2-fold dilutions qPCR ranged from 0.1 to 4.83. The CV in the 10-fold dilutions qPCR ranged from 0.10 to 7.69, being the highest values obtained for IL-12p40 and TGF-β (Table 3).

The mean FD in the 2-fold dilutions qPCR assays was lower than 1.29 for all cytokines except for IL-10 (FD = 2.59) (Table 4), which showed an irregular FD along the 2-fold and 10-fold dilution dynamic range. The mean FD in the 10-fold dilutions qPCR assays was lower than 1.97 for all the cytokines. The mean FD was higher in the 10-fold dilution than in the 2-fold dilutions qPCRs assays (Table 4). The FD increased in two-fold and ten-fold dilutions qPCR when less than 1000 template copies were used.



Primer	Tm	SD
IFN-α	77.01	0.29
IFN-γ	75.39	0.23
TNF-α	83.63	0.28
IL-12p35	79.12	0.24
IL-12p40	82.64	0.22
IL-10	82.87	0.22
TGF-β	83.11	0.27

Figure 2. **Dissociation curve analysis for each cytokine qPCR assay. The melting temperatures (Tm) for each cytokine are summarized in the table.**

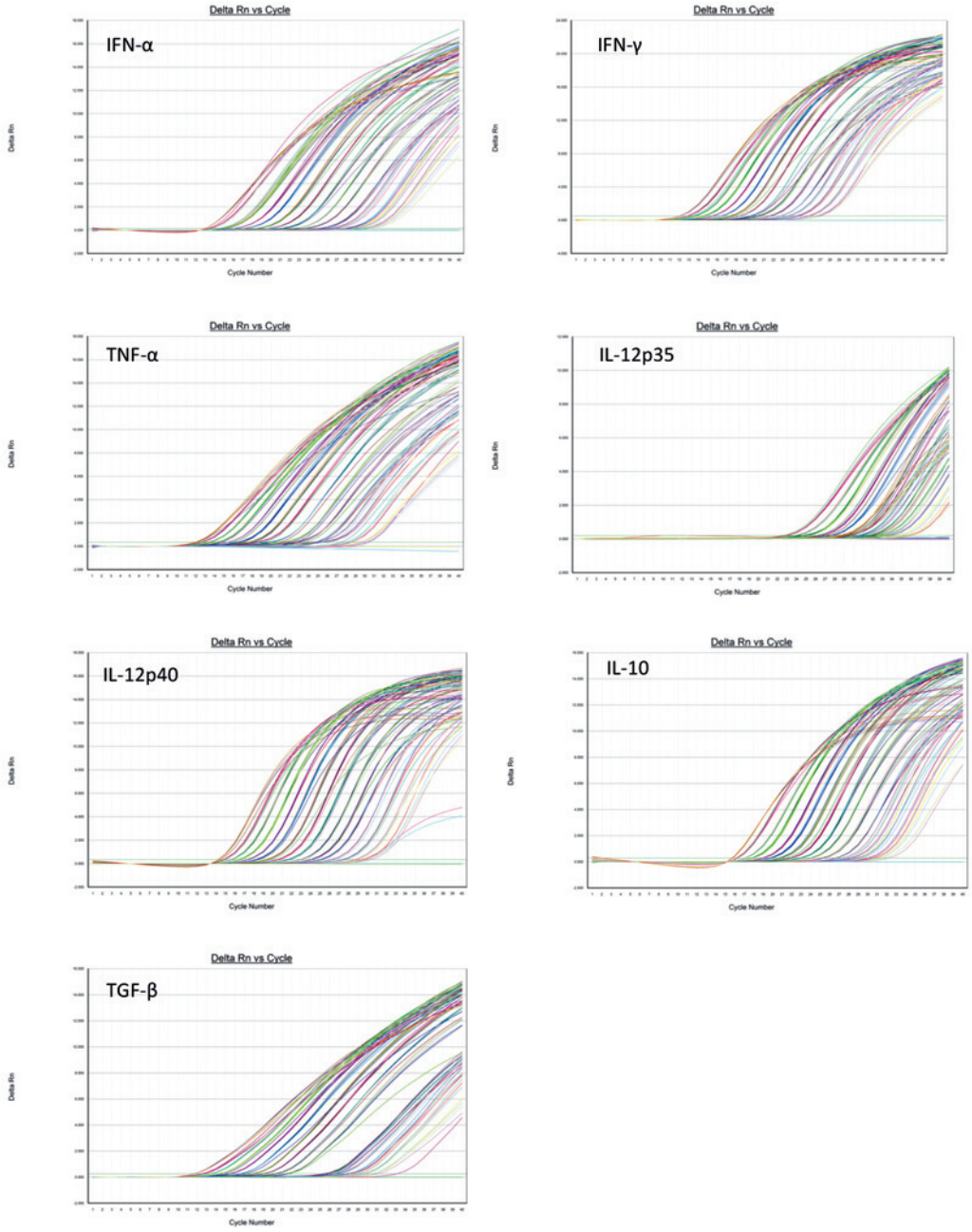


Figure 3. **Cytokine 2-fold dilutions qPCRs amplification plots in linear scale. No-template controls were included as negative controls. Template 2-fold dilutions ranged from 10^7 to 305 copies per µl.**

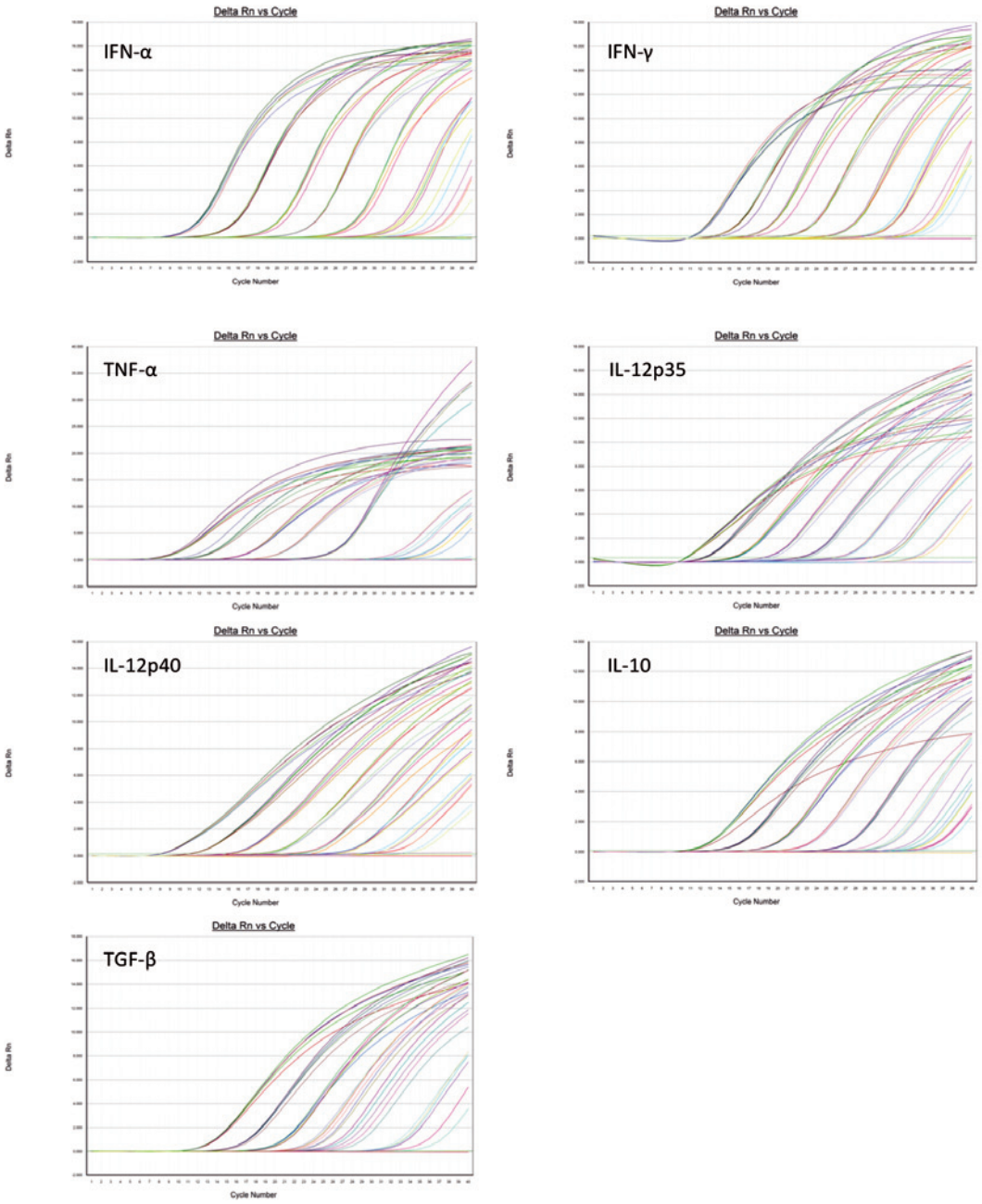


Figure 4. **Cytokine 10-fold dilutions qPCRs amplification plots in linear scale. No-template controls were included as negative control. Template 10-fold dilutions ranged from 10^8 to 10^1 copies per µl.**

Table 2.

	R^2		E		%E	
Cytokine	**1:2**	**1:10**	**1:2**	**1:10**	**1:2**	**1:10**
IFN-α	0,99	0,99	1,90	1,83	95,2	91,7
IFN-γ	0,99	0,99	1,92	1,90	96,25	95,25
TNF-α	0,99	0,99	2,00	1,68	100,45	84,2
IL-12 p35	0,97	0,99	1,91	1,97	95,55	98,75
IL-12 p40	0,95	0,99	1,82	1,97	91,1	98,9
IL-10	0,75	0,99	2,38	1,92	119,35	96,45
TGF-β	0,99	0,97	1,79	1,97	89,53	98,45

Correlation coefficient of the standard curve (R2), Efficiency (E) and percentage of the Efficiency (%E) of each qPCR assay,

Table 3.

Fold dilutions	cDNA copies	IFN-α	IFN-γ	TNF-α	IL-12 p35	IL-12 p40	IL-10	TGF-β
1:2	**10000000**	0,59	0,18	0,25	1,03	0,18	1,42	0,08
	5000000	0,44	0,17	1,53	0,14	0,18	0,14	0,95
	2500000	0,29	0,15	0,73	0,15	0,13	0,16	1,03
	1250000	0,34	0,64	0,54	0,19	0,27	0,46	0,42
	625000	0,16	0,26	0,32	0,45	0,12	0,50	0,10
	312500	0,42	0,55	0,24	0,39	0,66	0,82	0,21
	156250	0,87	0,56	1,15	0,23	0,15	0,90	0,55
	78125	0,38	1,09	0,11	0,28	0,12	2,49	0,33
	39062	0,42	0,38	0,68	0,41	0,18	2,17	0,79
	19531	0,16	0,17	0,91	0,58	0,41	2,53	0,15
	9765	0,26	0,45	0,47	0,50	0,37	1,96	0,82
	4882	0,31	0,18	0,14	0,21	0,74	0,34	0,67
	2441	0,55	0,75	0,43	0,38	0,50	1,33	0,53
	1220	0,38	1,36	0,51	0,71	0,78	0,95	0,92
	610	1,39	1,31	0,42	0,92	0,84	2,01	3,50
	305	0,99	1,99	0,74	0,71	3,62	2,52	0,96
1:10	**100000000**	0,47	0,40	1,23	0,54	0,56	1,44	0,72
	10000000	0,33	2,59	1,01	0,72	0,63	0,69	2,42
	1000000	1,44	2,73	1,44	0,97	2,08	0,56	2,35
	100000	0,13	1,31	0,99	1,99	3,77	0,57	3,11
	10000	1,11	0,31	2,85	1,77	5,56	0,78	6,64
	1000	1,04	0,78	2,13	1,244	3,05	2,47	7,10
	100	3,54	2,02	3,47	2,68	3,58	2,99	2,19
	10	0,1	2,32	1,88	1,02	7,69	2,74	3,50

Coefficient of Variation (CV) for 2-fold and 10-fold dilutions qPCR.

Table 4.

Fold dilutions	cDNA copies	IFN-α	IFN-γ	TNF-α	IL-12 p35	IL-12 p40	IL-10	TGF-β
1:2	**10000000**	1,11	1,03	1,04	1,23	1,03	4,68	1,01
	5000000	1,08	1,03	1,31	1,03	1,03	1,06	1,16
	2500000	1,01	1,03	1,14	1,03	1,02	1,07	1,19
	1250000	1,07	1,16	1,11	1,04	1,05	1,22	1,07
	625000	1,03	1,06	1,07	1,11	1,02	1,26	1,01
	312500	1,10	1,15	1,05	1,10	1,14	1,48	1,04
	156250	1,24	1,16	1,32	1,06	1,03	1,58	1,12
	78125	1,10	1,36	1,03	1,07	1,02	4,83	1,08
	39062	1,12	1,12	1,20	1,12	1,04	3,26	1,21
	19531	1,05	1,05	1,29	1,19	1,11	4,20	1,04
	9765	1,08	1,16	1,15	1,17	1,12	3,11	1,25
	4882	1,10	1,06	1,04	1,07	1,26	1,22	1,22
	2441	1,20	1,31	1,15	1,14	1,17	2,41	1,17
	1220	1,14	1,67	1,19	1,29	1,35	1,81	1,35
	610	1,68	1,68	1,16	1,42	1,34	3,49	3,19
1:10	**100000000**	1,08	1,06	1,09	1,08	1,08	1,23	1,11
	10000000	1,06	1,57	1,11	1,12	1,12	1,13	1,59
	1000000	1,41	1,81	1,23	1,23	1,23	1,13	1,76
	100000	1,03	1,42	1,19	1,63	1,63	1,17	2,43
	10000	1,45	1,10	1,96	1,71	1,71	1,30	1,72
	1000	1,50	1,34	1,91	1,55	1,55	2,59	1,72
	100	4,70	2,35	3,24	2,97	2,97	3,72	2,50
	10	1	2,74	1	1,60	1,60	3,53	1

Factor of Discrimination (FD) for 2-fold and 10-fold dilutions qPCRs.

DISCUSSION AND CONCLUSIONS

Cytokines play an important role in the immune response signalling cascade. The understanding of the complex interplay between pro- and anti-inflammatory phenomenons during host-pathogen interaction is still unmet and needs to be further characterized. Further understanding of the role of cytokines in the pathobiological mechanisms of respiratory swine diseases holds the key to the development of effective prophylactic and therapeutic strategies (Gómez-Laguna *et al*., 2013; Coussens *et al*., 2004)

RT-qPCR is a sensitive, specific, rapid, reproducible and reliable method for mRNA quantification (Ficko and Cernelc, 2005) and has been used frequently to measure the immune responses gaining popularity in vaccine immunology. In order to obtain meaningful re-

sults, optimized RT-qPCR assays must be available. However, many qPCR assays are poorly described and validation is lacking in published papers. In the current study, we validated the performance of qPCR assays for swine proinflammatory (IFN-α, IFN-γ, IL-12p35, IL-12p40 and TNF-α) and immunomodulatory (IL-10 and TGF-β) cytokines on cDNAs from pig tissues and on plasmids. Poor choice of reverse-transcription primers and optimization of their concentrations leads to inefficient assay performance (Bustin *et al*., 2009). The primers used in this study were selected from previously published papers, in order to produce an amplicon smaller than 150 bp which has been demonstrated to improve qPCR efficiency (Nolan *et al*., 2009). Furthermore, some manufacturer's recommendations indicate 300 bp as maximum amplicon size to ensure the efficiency (ABgene). Our results showed that each primer set produced unique PCR products, indicating that the primers are gene specific. In order to provide a sensitive and reliable tool to measure the pig proinflammatory and immunomodulatory response during experimental or naturally occurring respiratory disease we tested their capacity to be performed simultaneously with the same qPCR amplifying conditions. The obtained results suggested that these qPCR assays were specific, sensitive, robust and reproducible when they are performed under the same qPCR amplifying conditions.

Amplification efficiency is an important parameter to perform proper gene expression analysis (Bustin *et al*., 2009). Optimal PCR efficiencies are in the range between 1.9 and 2.1 (Nolan *et al*., 2006). Gene expression changes can be calculated using an absolute or relative quantification approach (Pfaffl, 2004). Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal reference transcript (Pfaffl, 2004). Relative quantification does not require standards with known concentrations, which makes it easier and faster compared to absolute quantification. To calculate the expression of a target gene in relation to an adequate reference control gene, calculations are based on the comparison of the Cq assuming that the E of the target gene and the reference gene are equal unless an E correction method (which implies to know the E of each target) is used (Pfaffl, 2004). Ignoring the differences between target and reference gene efficiencies may lead to miscalculation of gene expression even with the same starting amount of template. In the present study, we have observed that the different qPCR assays tested yielded different E values; all of them within the acceptable range between 1.9 and 2.1 at least in one of the two dilutions series tested (10-fold or 2-fold) as previously recommended (Nolan *et al*.,2006). This finding implies that methods that correct for different E should be applied in the relative gene quantification of these cytokines (Pfaffl, 2004).

The assays detected 100 copies of IFN-α, IFN-γ, IL-12p35, IL-12p40 and IL-10 input plasmid and 305 copies of TGF-β and TNF-α input plasmid. We experimentally demonstrate that these assays are sensitive enough to detect even low levels of cytokine gene expression in porcine tissues.

Despite no studies of validation of qPCR assays for porcine cytokines have been reported in the literature, the results obtained in the current study were comparable to the previously qPCR parameters published for human virus qPCR assays in which CV values were between 0.7 and 5.6% (Ruelle *et al*., 2004; Chapagain *et al*.,2006). The present validation results of CV indicated that the qPCR assays variability was lower than 3% along the 2 and 10-fold dynamic range for all cytokines, except for IL-12p40 and TGF-β 10-fold dilutions which CV was higher than 5% just in some dilutions. Therefore, we consider that the present qPCR assays are satisfactory for monitoring proinflammatory and

immunomodulatory swine responses. Although it was observed that fewer than 1000 template copies increased the FD, there was no correlation between template copy number and the CV values, indicating that the assays are robust with low template inputs.

To the author's knowledge no reference study about FD in qPCR are available regarding swine gene expression in the literature. The mean FD in 2-fold and 10-fold serial dilutions was always lower than 3, which could be considered optimal results because of their power to detect small differences in gene expression.

Importantly, we show that all the qPCR assays can be run simultaneously in one plate in separate wells under the same amplifying conditions. The qPCR platform running proinflammatory and immunomodulatory pig cytokines could be of significant value for several reasons. Firstly, the ability to evaluate cytokine responses with high sensitivity using only few mg of tissue means that sampling might take place in many more clinical settings where there is no need to kill animals since the organ samples could be easily obtained using an eco-guided punction. Secondly, the qPCR assay is highly flexible because nucleic acid samples can be stored at any stage after obtaining, and unlike direct assays, just as ELISPOT, cDNA may also be reproved for testing novel transcripts (Kasprovicz *et al*., 2011). We provide here evidence that the development of the tested qPCR assays is sensitive, specific, robust and highly useful in studying proinflammatory and immunomodulatory swine respiratory responses.

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