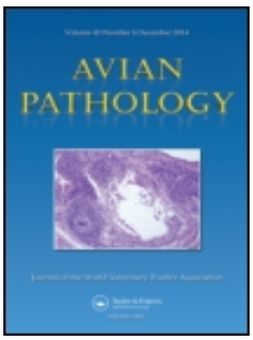

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Selection of reference genes for gene expression analysis by real-time qPCR in avian cells infected with infectious bronchitis virus

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Short title: Reference genes for qPCR in IBV studies

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Abstract (250 words max)

Infectious bronchitis virus (IBV) causes infectious bronchitis in poultry, a respiratory disease that is a source of major economic loss to the poultry industry. Detection and the study of the molecular pathogenesis of the virus often involves the use of real-time quantitative PCR assays (qPCR). To account for error within the experiments, the levels of target gene transcription are normalised to that of suitable reference genes. Despite publication of the MIQE guidelines in 2009, single un-tested reference genes are often used for normalization of

qPCR assays in avian research studies. Here we use the geNorm algorithm to identify suitable reference genes in different avian cell types during infection with apathogenic and pathogenic strains of IBV. We discuss the importance of selecting an appropriate experimental sample subset for geNorm analysis, and show the effect that this selection can have on resultant reference gene selection. The effects of inappropriate normalization on the transcription pattern of a cellular signalling gene, *AKT1*, and the interferon-inducible, *MX1*, were studied. We identify the possibility of the misinterpretation of qPCR data when an inappropriate normalisation strategy is employed. This is most notable when measuring the transcription of *AKT1*, where changes are minimal during infection.

Keywords

Infectious bronchitis virus, reference gene selection, Real-time quantitative PCR, geNorm, normalisation

Introduction

Infectious bronchitis is one of the most economically detrimental diseases to the poultry industry. The infection, caused by the gammacoronavirus, infectious bronchitis virus (IBV), initially establishes itself within the respiratory tract and often leads to secondary bacterial infections. The emergence of new strains and the lack of cross protection between them mean that effective vaccines that fully protect against IBV are difficult to develop. Early and quick detection of the virus is therefore important for the control of the disease. IBV has historically been detected via various methods including virus isolation in embryonated eggs, tracheal organ cultures or immunoassays. These methods however are expensive and time consuming and therefore, the use of broadly targeted multi-probe qPCR assays are more often used (Hewson et al., 2009; Muradrasoli et al., 2009). In these assays the transcription of strain

specific viral genes are measured and the levels of viral RNA are normalised to host reference genes. The selection of the most stable reference genes is important to compensate for any errors in sample preparation and processing. The study of the replication of infectious bronchitis virus, like many other viruses infecting non-model species, is often hindered by the absence of molecular reagents. However, with the design of primer and probes, qPCR is often used for the study of the molecular basis of viral replication. In this study we investigate the effects of inaccurate normalisation on two host genes. The interferon-induced *MX1* gene and *AKT1*, encoding for a cellular signalling protein. The transcription profiles of both genes are predicted to be different during infection, with *MX1* transcription altered by viral infection. On the contrary, activity of AKT1 is known to be modulated by phosphorylation at the protein level and therefore gene transcription is unlikely to be altered due to virus infection.

In a previous study, *GAPDH* and *UB* were identified as suitable reference genes in IBV-M41 infected SPF chickens (Fan et al., 2012). In the study, Fan et al provide an indication of potentially suitable reference genes in IBV infected tissues. There is, however, a need to identify the most stable reference genes for each individual experimental condition by the use of algorithms such as geNorm (Vandesompele et al., 2002), Normfinder (Andersen et al., 2004) or BestKeeper (Pfaffl et al., 2004), this is discussed in the MIQE guidelines published in 2009 (Bustin et al., 2009). Several studies have also investigated the implications of inappropriate normalisation (Dheda et al., 2005; Tricarico et al., 2002). Staines et al (2016) discuss the importance of reference gene selection and compare the different normalization methods available. It is now widely accepted that multiple reference genes must be used to normalize data, however many recent studies have recommended a set of reference genes for the study of gene expression in avian species (Bages et al., 2015; Borowska et al., 2016; Chapman et al., 2016; Nascimento et al., 2015; Olias et al., 2014; Yin et al., 2011), or

particular avian viral infections (W. Q. Fan et al., 2012; Li et al., 2005; Yang et al., 2013; Yue et al., 2010). Yang et al. (2013) recommend the use of *RPL30* and *SDHA* as reference genes for mRNA transcription analysis in chicken embryo fibroblasts (CEF) infected with avian leucosis virus. The stability of reference genes in CEFs infected with Newcastle disease virus was also investigated and *ACTB*, *HPRT1* and *HMBS* were recommended for use in such studies *(*Yin et al., 2011*)* . Whilst the majority of these publications discuss the implications of inappropriate normalisation methods, they recommend a set of reference genes identified as being suitable for a particular set of experimental data. These studies may give an indication as to appropriate reference genes for certain experimental conditions. However, these genes should be used as candidate reference genes only, and their stability be measured for each experimental condition independently. This strategy is not often employed in avian viral research. The present study discusses the design of geNorm analysis studies and the implications of inappropriate normalization on the measurement of host gene transcription during IBV infection of avian cells.

Materials and Methods

Cell culture and virus strains. Chick kidneys were prepared from 2 to 3 week old specific pathogen free (SPF) Rhode Island Red chickens. Primary chick kidney cells (CK cells) were prepared as described previously (Hennion & Hill, 2015). DF1 cells (Himly et al., 1998) were maintained in 1x DMEM (Sigma) supplemented with 10% FCS and 100U Pen/Strep.

IBV Beau-R is an apathogenic molecular clone of Beaudette-CK described previously (Casais et al., 2001). M4-CK is a pathogenic strain of IBV adapted to grow in CK cells, here referred to as M41(Darbyshire et al., 1979).

Sample preparation and experimental design. CK cells were infected with IBV-Beau-R (MOI 1), IBV-M41 (MOI 1) or mock infected. At 12 and 24 hours post infection the RNA was

extracted using RNeasy Mini kit (Qiagen). RNA was quantified using a Nanodrop (Thermo Scientific). One µg of RNA was reverse transcribed using Superscript III (Invitrogen) according to manufacturer's protocol including a DNase step and using random primers (Promega).

Selection of appropriate reference genes. SYBR Green qPCR was performed using Power SYBR Green Master Mix (ThermoFisher Scientific). Primers were used at a final concentration of 0.8 µM and sequences are shown in Table 1 (Staines et al., 2016). The qPCR was run on a 7500 Fast Real Time System (Applied Biosystems) with the following cycle profile: 95 °C for 10 mins and then 40 cycles at 95 °C for 15 sec and 60 °C for 30 sec. A dissociation step was included for melt curve analysis. The raw CT values were analyzed using the geNorm algorithm through the qbase+ software (Biogazelle).

Measurement of gene transcription by qPCR. TaqMan qPCR was performed using TaqMan® Fast Universal PCR 2x Master Mix (ThermoFisher Scientific). Primers were used at 500nM and hydrolysis probes at 125nM final concentrations **(**primer and probe sequences are shown in Table 2 (Staines et al., 2016)). The qPCR was run on a 7500 Fast Real Time System (Applied Biosystems) with the following cycle profile: 95 °C for 1 mins and then 40 cycles at 95 °C for 10 sec and 60 °C for 30 sec. IBV 5′UTR primer and hydrolysis probe sequences were as follows: IBV5′ Forward, 5′-GCTTTTGAGCCTAGCGTT-3′; IBV5′ Reverse 5′-GCCATGTTGTCACTGTCTATTG-3′; and IBV 5′ probe, 5′ (FAM)-CACCACCAGAACCTGTCACCTC-(TAMRA)-3′ (Maier et al., 2013).

Results

Selection of samples for geNorm analysis. CK cells were infected with a pathogenic (M41) and apathogenic (Beau-R) strain of IBV and RNA collected at 12 and 24 hpi. The viral genome copy number was quantitated by qPCR using a standard curve (Figure 1). Viral

genome copy number was normalised to a mock infected sample at each time point. An increase in genome copies can be seen from 12 to 24 hpi with similar levels of Beau-R and M41 genome being produced at each time point. A SYBR green qPCR was then performed using primer sets previously described by Staines et al (2016). The stability factors of candidate reference genes were established by imputing CT values of gene transcription into the geNorm algorithm. Of the three replicates, one from each experimental subset was chosen for testing. The relative stability of 9 candidate genes was ranked by geNorm M value, with the more stable genes having the lowest value. The stability of the candidate reference genes in CK cells infected with Beau-R and M41 are shown (Figure 2 (a)). The reference genes were ranked according to their stability, from lowest to highest as; *28s rRNA, TBP*, *RPLP0*, *RPL13*, *GAPDH*, *HPRT1*, *PGK1*, *HMBS* and *ACTB*. The geNorm V values (not shown) recommend the optimal number of reference genes to be used. In this case the results of the algorithm suggest that the addition of the third most stable reference gene, *PGK1,* to *ACTB* and *HMBS* does not significantly increase the stability of the reaction. Therefore it is recommended that *ACTB* and *HMBS* be used as reference genes in this experimental set up.

The importance of using an appropriate subset of data was investigated by removing sections of data from the analysis. Figure 2 (b) and (c) show the candidate reference genes ranked according to stability in CK cells infected with Beau-R and M41 at 12 hpi or 24 hpi, respectively. The stability of the reference genes changes depending on which subset of data is used. The most suitable reference genes at 12 hpi are *HMBS* and *RPL13* whereas at 24 hpi they are *ACTB* and *HPRT1*. Differences in the stability of reference genes when CK cells were infected with the different strains of IBV, Beau-R (Figure 2 (d)) and M41 (Figure 2 (e)) were investigated. Here again, the most appropriate reference genes are different between the different experimental setups with *HMBS* and *ACTB* being more stable in CK cells infected

with Beau-R and *HPRT1* and *TBP* being the most suitable genes in CK cells infected with M41.

In addition to the primary CK cells, the apathogenic strain Beau-R is able to infect the continuous avian cell line, DF1 cells. DF1 cells were infected with Beau-R and RNA extracted at 12 and 24 hpi. Viral genome copy number was quantified by qPCR and an increase in viral genome copy number can be seen between 12 and 24 hpi (Figure 3). SYBR green qPCR assays were performed using the primer sets described in Table 1, and the raw data analyzed using the geNorm algorithm. The reference genes in order of stability in DF1 cells infected with Beau-R at 12 and 24 hpi were; *28s rRNA*, *GAPDH*, *ACTB*, *TBP*, *RPLP0*, *PGK1*, *HPRT1*, *HMBS* and *RPL13* (Figure 4 (a)). The geNorm V values (not shown) recommend the use of the first two reference genes for these experimental samples, *HMBS* and *RPL13*. Once more, the effect of selecting a smaller subset of the data was investigated. The two most suitable reference genes in the 12 hpi samples of DF1 cells infected with Beau-R were *HPRT1* and *HMBS* where as *ACTB* was the least stable (Figure 4 (b)). At 24 hpi the most stable reference genes were *PGK1* and *GAPDH* (Figure 4 (c)). The variation in the stability of reference genes is consistent across the cell types, virus strain and time points examined.

Effect of inappropriate normalisation. The selection of appropriate reference genes has been discussed at length as being important for the accurate analysis of gene expression levels. The two genes with the highest stability measure in DF1 cells infected with Beau-R at 12 and 24 hpi were found to be *HMBS* and *RPL13*. The effect of inappropriate normalisation on studies of gene transcription was investigated. The transcription of avian *AKT1* was compared to that of the interferon-inducible avian *MX1* when normalised using different reference genes (Figure 5). The most suitable reference genes as identified by geNorm (*RPL13* and *HMBS*)

were compared to the use of single, commonly used, reference genes with low stability values, *ACTB* and *28s rRNA*. Figure 5a and b respectively show a significant change, calculated by unpaired t-test, in transcription of both *AKT1* (*P*=0.0025) and *MX1* (*P* =0.0382) over time during infection, when normalised to *RPL13* and *HMBS*. The use of a single reference gene changes the significance of the data. This is most notably different in the *AKT1* data where changes in transcription are smaller than those of *MX1* during infection. For example changes *AKT1* transcription normalised to *RPL13* and *HMBS* has *P* =0.0025 (Figure 5 (a)) whereas, when the same Ct values are normalised to *ACTB*, *P* =0.3170 (Figure 5 (b)). However, the change in the significance of *MX1* transcription is not as different, where *P* =0.0382 when normalised to *RPL13* and *HMBS* (Figure 5 (b)) compared to *P* =0.0903 when normalised to *ACTB* (Figure 5 (d)). The use of *28s rRNA* as a single reference gene was also investigated. The change in significance of *AKT1* gene expression (from *P* =0.0025 to 0.0213) was seen (Figures 5 (a) and 5 (e)), however this change is less notable for *MX1* transcription (Figures 5 (b) and 5 (f)).

Discussion

Limitations that prevent the use of reference gene stability analysis include lack of sufficient sample, time and cost of reagent. A cross sectional subset of samples is therefore often used, and the choice of samples has an impact on the outcome of the calculation of reference gene stability. In this study the transcription of candidate reference genes in one batch of three replicates was analyzed, as viral genome copy number had previously been found to be consistent throughout the samples. We were interested to investigate whether selecting a smaller subset of the samples would have had an effect on the stability measure of the reference genes. The stability changed depending on the cell type, strain of virus and the time at which the RNA was collected (Figures 2 and 4). Most notably the stability of *TBP* was

found to be low in CK infected samples except in CK cells infected with M41 at 12 and 24 hpi. Also, a commonly used reference gene, *ACTB*, was found to be one of the more stable genes in CK cells however, that stability was not observed in DF1 cells. Ribosomal RNA has historically been used in avian studies as a reference gene. However, in this study we have found it to be the least stable reference gene out of the candidates in both CK and DF1 cells infected with IBV. This suggests that the selection of an appropriate subset of experimental samples is an important step in accurate normalisation of mRNA transcription levels.

There has been a recent surge in the publication of reference gene stability papers, most of which recommend a set of four to six reference genes for use in a particular experimental setup. Whilst the importance of using multiple reference genes is recognized in these studies, the suggestion that these reference genes may have uniform transcription across different experimental samples performed in different laboratories is misleading. Despite significant improvement in the design and publication of qPCR studies in model species, single reference genes are still used for normalisation of target genes in non-model species such as *Gallus gallus.* We therefore investigated variations in the transcription of two avian genes when normalised to geNorm selected reference genes, *HMBS* and *RPL13* and also *ACTB* and *28s rRNA*. The mRNA expression of *AKT1* was measured along with that of *MX1*, which is known to change during infection with IBV (Cong et al., 2013). We found a significant change in transcription levels of both *AKT1* and *MX1* mRNA during infection from 12 to 24 hpi when normalised correctly to the selected reference genes (Figures 5 (a) and 5 (b)). This correlated with an increase in copies of IBV genome present (Figure 3). When the same raw data was normalised to *ACTB* the statistical significance of the variation was lost and the transcription of mRNA would be interpreted as not changing over time during IBV infection (Figures 5 (c) and 5 (d)). The most notable changes were in the transcription of *AKT1* mRNA (Figures 5(a),

(c), (e)). This highlights that data sets showing small changes in mRNA levels are more susceptible to inappropriate normalisation strategies than those with large changes, such as in the case of *MX1*. Nonetheless, our study clearly demonstrates the importance of correct selection of reference genes for robust analysis of experimental data.

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Disclosure statement

The authors declare no conflict of interest.

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Table 1. Primer sequences and accession numbers of candidate reference genes used for geNorm analysis (Staines et al., 2016)

Gene		Sequence	Accession Number
ACTB	Forward	5'-CAGGTCATCACCATTGGCAAT-3'	NM_205518
	Reverse	5'-GCATACAGATCCTTACGGATATCCA-3'	
HMBS	Forward	5'-GGTTGAGATGCTCCGTGAGTTT-3'	XM_417846
	Reverse	5'-GGCTCTTCTCCCCAATCTTAGAA-3'	
HPRT1	Forward	5'-TGGTCAAAAGAACTCCTCGAAGT-3'	NM_204848
	Reverse	5'-TGTAATCGAGGGCGTATCCAA-3'	
PGK1	Forward	5'-GTTTATGTCAATGATGCTTTTGGAA-3'	NM_204985
	Reverse	5'-GCCTTTGCAAAATAATCCAGTTCT-3'	
RPL13	Forward	5'-TCGTGCTGGCAGAGGATTC-3'	NM_204999
	Reverse	5'-TCGTCCGAGCAAACCTTTTG-3'	
RPLP0	Forward	5'-TTGTTCATCACCACAAAGATT-3'	NM_204987
	Reverse	5'-CCCACTTTGTCTCCGGTCTTAA-3'	
TBP	Forward	5'-CTTCGTGCCCGAAATGCT-3'	NM_205103
	Reverse	5'-GCGCAGTAGTACGTGGTTCTCTT-3'	
28s rRNA	Forward	5′-GGCGAAGCCAGACCAAACT-3′	X59733
	Reverse	5′-GACGACCGATTTGCACGTC-3′	
GAPDH	Forward	5′-GGTGGTGCTAAGCGTGTTA-3′	X01578
	Reverse	5′-CCCTCCACAATGCCAA-3′	

Table 2. Primer and probe sequences and accession numbers of *AKT1* and *MX1* genes as well as reference genes.

Gene		Sequence	Accession Number
AKT1[a]	Forward	5'-TCACGGCATCCATTCTTAAACA-3'	NM_205055
	Reverse	5'-CTTCAGAAAATACACACTCTCTC-3'	
	Probe	5‘-AAAACAACTCCCCTCCGTTAGCATACTCCA(BHQ)-3'	
MX1[b]	Forward	5'-CACTGCAACAAGCAAAGAAGGA-3'	NM_204609.1
	Reverse	5'-TGATCAACCCCACAAGGAAAA-3'	
	Probe	5'(FAM)-ACAAAGCACACACCCAACTGTCAGCG-(TAMRA)-3'	
HMBS	Forward	5'-GGTTGAGATGCTCCGTGAGTTT-3'	XM_417846
	Reverse	5'-GGCTCTTCTCCCCAATCTTAGAA-3'	
	Probe	5'-(FAM)-CCTGACCTCTGCTTTGAGATTGTTGCCA-(TAMRA)-3'	
RPL13	Forward	5'-TCGTGCTGGCAGAGGATTC-3'	NM_204999
	Reverse	5'-TCGTCCGAGCAAACCTTTTG-3'	
	Probe	5'-(FAM)-TAATGCCCGCCAGTTTAAGCTCTTCTAGGC-(TAMRA)-3'	
ACTB	Forward	5'-CAGGTCATCACCATTGGCAAT-3'	NM_205518
	Reverse	5'-GCATACAGATCCTTACGGATATCCA-3'	
	Probe	5′ -(FAM)-CACAGGACTCCATACCCAAGAAAGATGGC-(TAMRA)-3′	
28s rRNA	Forward	5′-GGCGAAGCCAGACCAAACT-3′	X59733
	Reverse	5′-GACGACCGATTTGCACGTC-3′	
	Probe	5′-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′	

[a]AKT1 primers and probe designed and produced by Primerdesign Ltd., UK
[b]MX1 primer and probe designed by William Mwangi, The Pirbright Institute, UK

Figure Captions

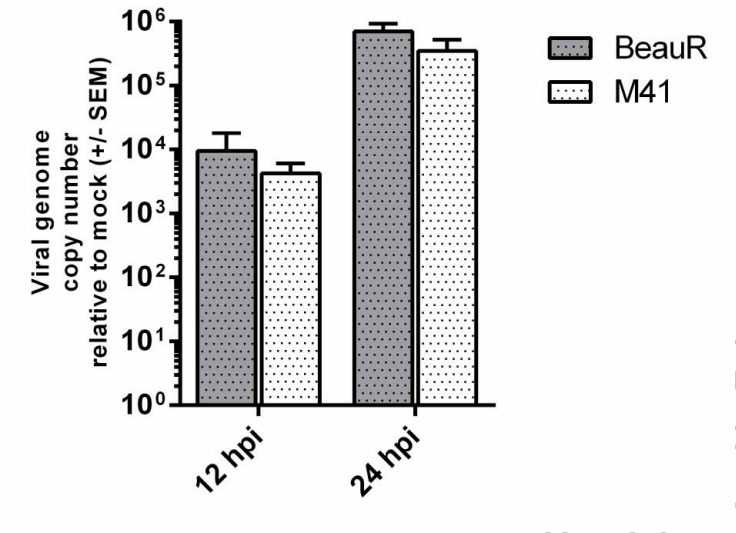
Figure 1. Viral genome copy number in CK cells infected with IBV. CK cells were infected with IBV Beau-R and M41 strains and at 12 and 24 hpi cells were lysed and RNA extracted. Viral genome was amplified by qPCR of the 5′UTR region of the genome. CT values were compared to a standard curve to calculate genome copy number. Copy number was normalised to control mock levels and the average of three replicates is plotted as viral genome copy number (±SEM).

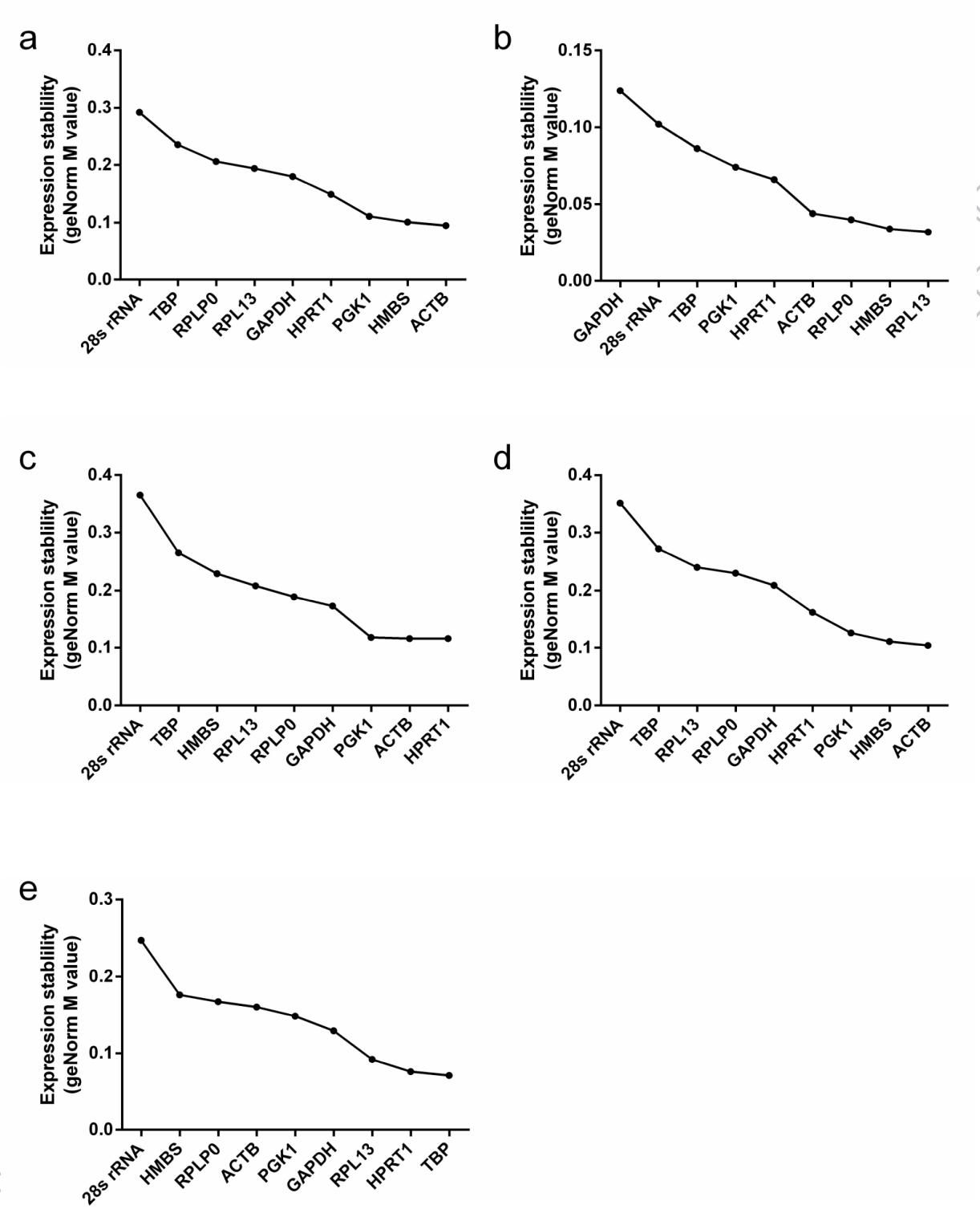
Figure 2. Transcriptional stability of candidate reference genes in IBV infected CK cells. CK cells were infected with IBV Beau-R and M41 and at 12 and 24 hpi cells were lysed and RNA extracted. Candidate reference gene mRNA was amplified by SYBRgreen qPCR. The transcriptional stability of the candidate reference genes is calculated by inputting CT values into the geNorm algorithm. The geNorm algorithm calculates the M value, a representation of the stability of the reference genes. The lower the M value, the more stable the gene between the experimental samples. (a) The geNorm M values of candidate reference genes in CK cells infected with mock, BeauR or M41 at 12 and 24 hpi. GeNorm M values of candidate reference gene transcription in CK cells infected with Beau-R and M41 at 12 hpi (b) or 24 hpi (c). M values of candidate reference gene transcription in CK cells infected with Beau-R (d) or M41 (e) at 12 and 24 hpi.

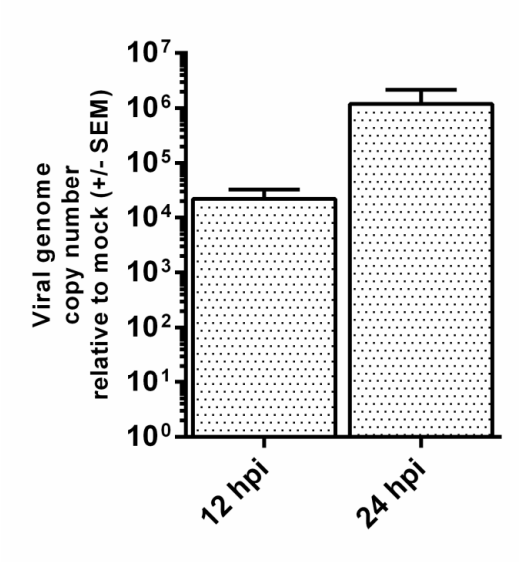
Figure 3. Viral genome copy number in DF1 cells infected with Beau-R. DF1 cells were infected with IBV Beau-R and at 12 and 24 hpi cells were lysed and RNA extracted. Viral genome present was amplified by qPCR of the 5′UTR region of the genome. CT values were compared to a standard curve to calculate genome copy number. Copy number was normalised to control mock levels and the average of three replicates is plotted as viral genome copy number (±SEM).

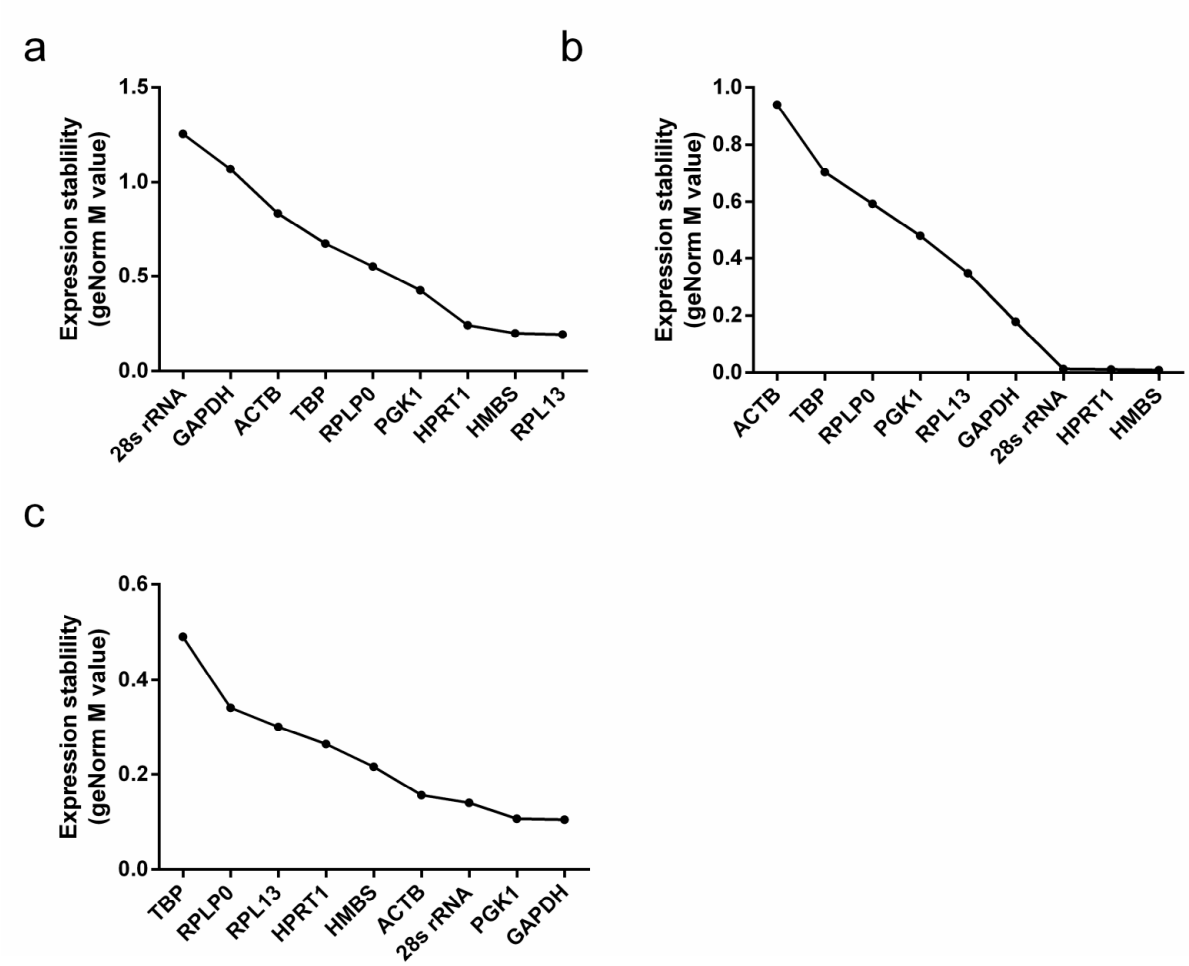
Figure 4. Transcriptional stability of candidate reference genes in IBV infected DF1 cells. DF1 cells were mock infected or infected with IBV Beau-R and at 12 and 24 hpi cells were lysed and RNA extracted. Candidate reference gene mRNA was amplified by SYBRgreen qPCR. The transcriptional stability of the candidate reference genes is calculated by entering CT values into the geNorm algorithm. The geNorm algorithm calculates the M value which is a representation of the transcriptional stability of the candidate reference genes. The lower the M value, the more stable the gene. (a) The M values of candidate reference genes in DF1 cells infected with mock and Beau-R at 12 and 24 hpi. The M values of candidate reference gene transcription in DF1 cells infected with mock or Beau-R at 12 (b) or 24 hpi (c).

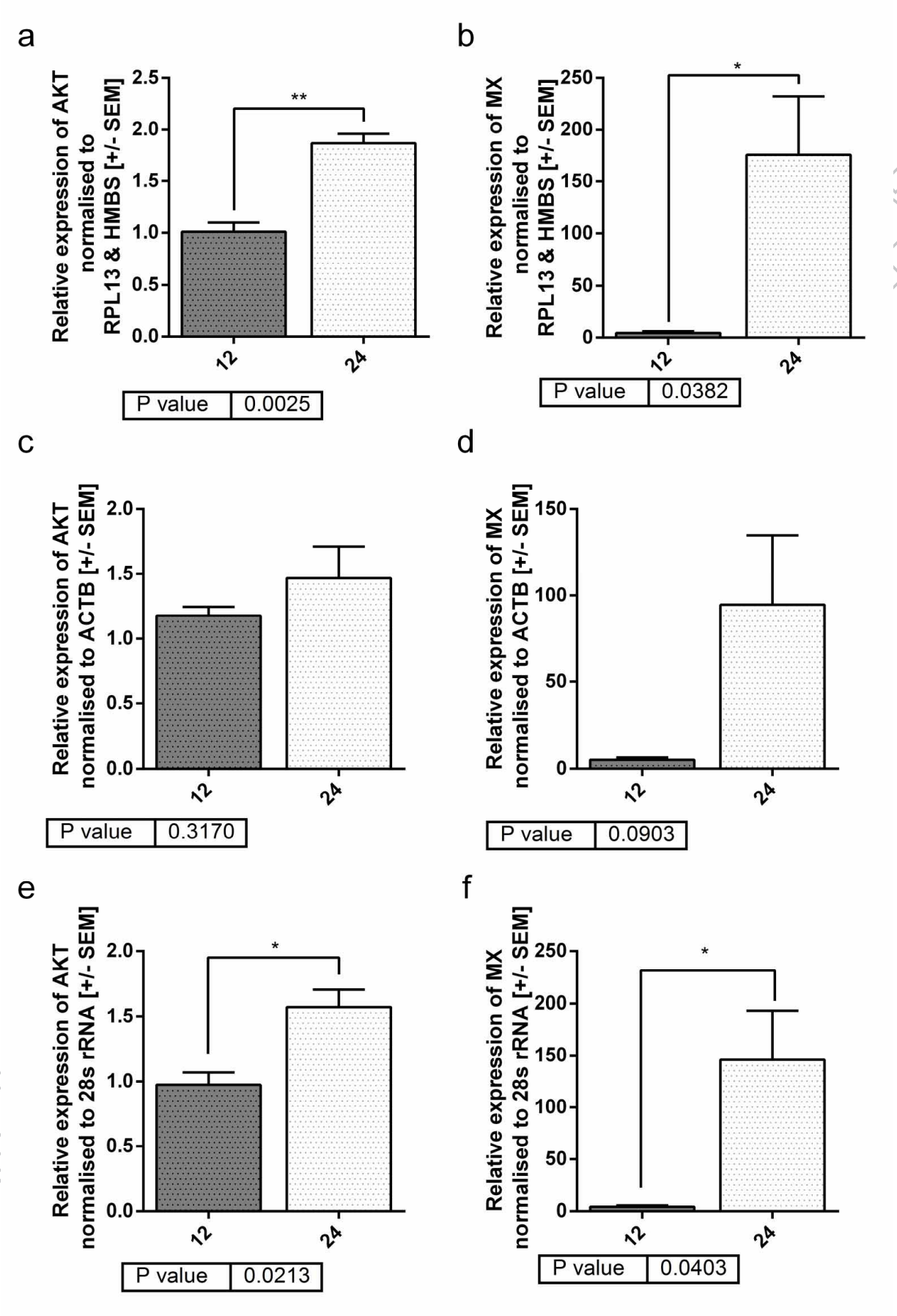
Figure 5. Transcription of *AKT1* and *MX1* genes normalised to various reference genes. DF1 cells were infected with mock or Beau-R and at 12 and 24 hpi cells were lysed and RNA extracted. *AKT1* and *MX1* mRNA was amplified by qPCR and transcription calculated using ΔΔCT and normalising to *RPL13* and *HMBS*, *ACTB* or *28s rRNA*. (a) *AKT1* transcription normalised to *RPL13* and *HMBS*. (b) *MX1* transcription normalised to *RPL13* and *HMBS*. (c) *AKT1* transcription normalised to *ACTB*. (d) *MX1* transcription normalised to *ACTB*. (e) *AKT1* transcription normalised to *28s rRNA*. (f) *MX1* transcription normalised to *28s rRNA*. *P* values calculated by two-tailed t-test.




a
Expression stablility (geNorm M value)
0.4
0.3
0.2
0.1
0.0
28s rRNA
TBP
RPLP0
RPL13
GAPDH
HPRT1
PGK1
HMBS
ACTB
b
Expression stablility (geNorm M value)
0.15
0.10
0.05
0.00
GAPDH
28s rRNA
TBP
PGK1
HPRT1
ACTB
RPLP0
HMBS
RPL13
c
Expression stablility (geNorm M value)
0.4
0.3
0.2
0.1
0.0
28s rRNA
TBP
HMBS
RPL13
RPLP0
GAPDH
PGK1
ACTB
HPRT1
d
Expression stablility (geNorm M value)
0.4
0.3
0.2
0.1
0.0
28s rRNA
TBP
RPL13
RPLP0
GAPDH
HPRT1
PGK1
HMBS
ACTB
e
Expression stablility (geNorm M value)
0.3
0.2
0.1
0.0
28s rRNA
HMBS
RPLP0
ACTB
PGK1
GAPDH
RPL13
HPRT1
TBP




a
Expression stability (geNorm M value)
1.5
1.0
0.5
0.0
28s rRNA
GAPDH
ACTB
TBP
RPLP0
PGK1
HPRT1
HMBS
RPL13
b
Expression stability (geNorm M value)
1.0
0.8
0.6
0.4
0.2
0.0
ACTB
TBP
RPLP0
PGK1
RPL13
GAPDH
28s rRNA
HPRT1
HMBS
c
Expression stability (geNorm M value)
0.6
0.4
0.2
0.0
TBP
RPLP0
RPL13
HPRT1
HMBS
ACTB
28s rRNA
PGK1
GAPDH


a
Relative expression of AKT normalised to RPL13 & HMBS [+/- SEM]
2.5
2.0
1.5
1.0
0.5
0.0
**
12
24
P value
0.0025
b
Relative expression of MX normalised to RPL13 & HMBS [+/- SEM]
250
200
150
100
50
0
*
12
24
P value
0.0382
c
Relative expression of AKT normalised to ACTB [+/- SEM]
2.0
1.5
1.0
0.5
0.0
12
24
P value
0.3170
d
Relative expression of MX normalised to ACTB [+/- SEM]
150
100
50
0
12
24
P value
0.0903
e
Relative expression of AKT normalised to 28s rRNA [+/- SEM]
2.0
1.5
1.0
0.5
0.0
*
12
24
P value
0.0213
f
Relative expression of MX normalised to 28s rRNA [+/- SEM]
250
200
150
100
50
0
*
12
24
P value
0.0403