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Validation of Reference Genes for Real-Time Quantitative PCR (qPCR) Analysis of *Avibacterium paragallinarum*

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Abstract

Real-time quantitative reverse transcription PCR (qRT-PCR) offers a robust method for measurement of gene expression levels. Selection of reliable reference gene(s) for gene expression study is conducive to reduce variations derived from different amounts of RNA and cDNA, the efficiency of the reverse transcriptase or polymerase enzymes. Until now reference genes identified for other members of the family *Pasteurellaceae* have not been validated for *Avibacterium paragallinarum*. The aim of this study was to validate nine reference genes of serovars A, B, and C strains of *A. paragallinarum* in different growth phase by qRT-PCR. Three of the most widely used statistical algorithms, geNorm, NormFinder and ΔCT method were used to evaluate the expression stability of reference genes. Data analyzed by overall rankings showed that in exponential and stationary phase of serovar A, the most stable reference genes were *gyrA* and *atpD* respectively; in exponential and stationary phase of serovar B, the most stable reference genes were *atpD* and *recN* respectively; in exponential and stationary phase of serovar C, the most stable reference genes were *rpoB* and *recN* respectively. This study provides recommendations for stable endogenous control genes for use in further studies involving measurement of gene expression levels.

Introduction

Infectious coryza is an acute upper respiratory tract disease of chickens. This disease is of worldwide economic significance and affects both broiler and layer flocks, manifested primarily as a drop in egg production (10±40%) in layer flocks and retardation of growth due to decreased feed and water consumption in breeder and broiler flocks. The most common clinical signs are nasal discharge, conjunctivitis, facial oedema, lacrimation, anorexia, and diarrhea [1].

The causative agent of infectious coryza is Avibacterium paragallinarum (*A. paragallinarum*) [2], and *A. paragallinarum* is classified into three serovars: A, B, and C according to the Page schemes [3]. It is widely accepted that the three Page serovars represent distinct

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"immunovars," since inactivated vaccines based on any one Page serovar provide no protection against the other two Page serovars [4]. However, little is known about the differences among these three serovars, either the genes defining each serovar or the expression of these genes.

Real-time quantitative reverse transcription PCR (qRT-PCR) is a robust and sensitive method for measurement of gene expression and characteraization of gene regulation. In most qPCR studies, internal reference genes are used to eliminate sample-to-sample variations that may arise due to test variation including differences in cell numbers and efficiency of RNA isolation and reverse transcription [5]. Since "housekeeping" metabolism of prokaryotes is highly variable depending on experimental procedures [6], selection of reference genes is crucial for the accuracy of a qRT-PCR test. Once the reference genes are selected, any changes in target gene expression can be expressed in relation to those of the reference genes. A single gene is often selected as the reference gene but Vandesompele et al [7] suggested that multiple carefully selected housekeeping genes were recommendable and more suitable for accurate normalization.

Development of an effective qPCR for defining gene expression in serovars A, B, and C of *A*. *paragallinarum* is urgently needed. However, information concerning reference genes as candidates for a qPCR against *A*. *paragallinarum* is very limited, primarily due to a lack of understanding the genome organization of serovars A, B, and C of *A*. *paragallinarum*. In this study, nine candidate reference genes encoding 16S ribosomal subunit (*16S rRNA*), the DNA gyrase subunit A (*gyrA*), theβ-subunit of RNA polymerase (*rpoB*), the glucose-6-phosphate isomerase (*pgi*), the DNA repair protein (*recN*), the translation initiation factor 2 (*infB*), the DNA gyrase subunit B (*gyrB*), the β-subunitof the ATP synthase (*atpD*) and the Mn-dependent superoxide dismutase (*sodA*), respectively, were chosen for validating the reference genes for qPCR of *A*. *paragallinarum*. The majority of these genes were recognized as housekeeping genes in the family of *Pasteurellaceae* and used in phylogenetic analysis [8–13]. Moreover, five of the nine genes (including 16S rRNA, gyrA, rpoB, *atpD* and gyrB) were also used for qRT-PCR normalization in *Actinobacillus suis* and *Haemophilus ducreyi* [14,15].

To date, no study has systematically investigated reference genes for A. paragallinarum. In order to verify the stable expression genes and determine whether they are suitable for normalization of qPCR data for *A*. *paragallinarum*, we performed molecular biological analysis of their expression stability. The objective of this work was to validate internal reference genes for a qRT-PCR of serovar A, B, and C strains of *A*. *paragallinarum*. The expression of nine reference genes was examined during different growth phase. The results of this study will be helpful for gene expression normalization of qPCR in serovars A, B, and C of *A*. *paragallinarum*.

Materials and Methods

Bacterial cultures and sample processing

The three reference strains 221, 0222, and Modesto of the *A*. *paragallinarum* serovar A, B, and C respectively were kindly provided by Dr. Pat Blackall, University of Queensland, Australia. Tryptic Soya Broth (TSB) and Tryptic Soya Broth Agar (TSA), supplemented with 10% (v/v) chicken serum and 0.0025% (w/v) reduced Nicotinamied adenine dinucleotide (NAD) were used for propagation and maintenance of these three strains. The cultures were grown in a shaking incubator at 37˚C. Broth Cultures of serovar A, B and C were monitored by OD600 measurement every 40 min, and samples were harvested from the exponential and stationary phase by centrifugation, followed immediately resuspended in RNAlater (Ambion, Carlsbad, CA). Samples were then stored at 4˚C until the test.

Table 1. Information of the primers and corresponding candidate reference genes.

Gene	Accession No.	Correlation (r^2)	Forward (F) and Reverse (R) Primers	Efficiency %	Amplicon length
gyrB	NZ_LAEN01000056.1	0.989	F:CAACTTCATCGCCCATTAGG;R:GGGAGAAATGAACCCAGAAC	88.2	191
recN	JN592546.1	0.981	F:AGCTTGCTCTACCGCACAAT;R:CTGGCTTCTTGCACCTGAAT	102.1	113
rpoB	NZ_AFFP02000004.1	0.994	F:GCTTAATGCCGCTTCACCTA;R:AGCGTGTGGTGCAAGAAGAT	99.1	131
infB	EU350938.1	0.999	F:GCCAGTTGCTACCATTTTGG;R:AGCCTAGCACTTCCACAGGA	97.3	155
pgi	JN592536.1	0.999	F:GGAAAGGCTACACAGGCAAA;R:AACACAAGGGTGGTTTCTGG	96.7	196
sodA	DQ005620.1	0.999	F:TTAGCAGAAGTGCCAGCAGA;R:GCTTCCACAGAACCGAAATC	92.7	155
atpD	AF326327.1	0.993	F:TCCCACAAGATGCAGTACCA;R:CCCACTGGAACAGAAATTGG	112.0	181
gyrA	NZ_AFFP02000003.1	0.993	F:AGTGAGCGTAACGGCAAAGT;R:ATGTCCGATTCTTCGTCGTC	98.7	218
16S rRNA	KF280244.1	0.997	F:AGGCCTTCGGGTTGTAAAGT;R:CGGGGATTTCACATCTCACT	99.2	201

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Primer design and validation

Primers were designed from GenBank sequences with the aid of primer analysis software Primer3plus (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi; Version 2.4.0) [16,17]. Then confirmed through DNAMAN and NCBI/Primer-BLAST. Characteristics of the primers are listed in Table 1. The length of the amplicons were kept between 100–250 bp as much as possible for optimal amplification efficiency. The effectiveness of the primers was confirmed by conventional PCR and product size observed by electrophoresis on 1.5% agarose gels. All nine primers produced single amplification products as expected (data not show).

RNA extraction and cDNA synthesis

Total RNA was extracted from 0.5 ml aliquot of bacterial samples collected at different growth phase using Trizol RT extraction system (Invitrogen, carlsbad, CA) following the manufacturer's instruction. The extracted RNA was re-suspended in DEPC-treated water (Life Technologies) and the concentration and purity of RNA were determined by NanoDrop1000® spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA). RNA samples with 260nm/280nm ratio between 1.9 and 2.1 were prepared in equimolar aliquots for further tests. cDNA was synthesized from 200 ng of each RNA sample [18] using a Reverse Transcription Kit (Tiangen, China). Prior to cDNA synthesis, genomic DNA (gDNA) in the RNA samples were removed by incubation with a gDNA buffer at 42˚C for 3 min as described in RNA reverse transcription kit (Tiangen, China). Reverse transcription reactions were performed in a MasterCycler ® Gradient Thermal Cycler under the following conditions: 42˚C for 15 min, 95˚C for 3 min. The cDNA samples were placed immediately on ice at the end of the reactions and then stored at -20˚C for later use.

Real-time quantitative PCR

Real-time quantitative PCR (qRT-PCR) was performed with Bio-rad IQ5 (Bio-Rad, Hercules, CA) using 2X SYBR Green iTaq mixture (Tiangen, China) in a total reaction volume of 12.5 ul. The reaction mixture consisted of: 6.25 ul of 2X SYBR Green iTaqmixture, 0.25 ul forward/reverse primer mix with an initial concentration of 10 uM, 1 ul of cDNA (1:2 dilution) and DEPC-treated water added to12.5 ul. Each sample was tested in triplicate. The cycling condition was as follows: 3 min denaturation at 95˚C, followed by 40 cycles at 94˚C for 40 s, 56/58˚C for 40 s and 72˚C for 40 s.

Data and statistical analysis

Cycle threshold (CT) values, also known as Cq recommended by Bustin [19], were recorded for all qPCR reactions. Two of the most widely used statistical algorithms, geNorm v3.5 and NormFinder [20,21] were used to evaluate the expression stability of reference genes. The comparative ΔCT method [22] was used to rank candidate reference genes, with the lowest standard deviation considered to signify the highest stability. The geNorm algorithm determines the most stable combination of reference targets based on the geometric mean of the most stable control genes to generate a stability value (M). The goal of the alanysis was to choose two or more reference genes to obtain more reliable quantitative results according to the pairwise variance analysis of normalization factor ($V_{n/n+1}$). NormFinder Excel applet, as a similar calculation method as geNorm and also based on relative expression levels, was used to assess reference gene stability based on both intra- and inter- group variations. NormFinder was used to identify genes with the lowest standard deviation (SV) as an indication for highest stability. The comparative ΔCT method was subsequently used to further evaluate gene expression stability. This method compares the relative expression of pairs of genes within each treatment and selects the most stable reference gene by assessing expression stability based on standard deviation derived from CT values. If the ΔCT values between pairs of genes remain constant for all samples tested, it means these two genes are either stably expressed or co-regulated. However, if the ΔCT values vary logarithmically, as reflected by higher standard deviations, it indicates that one of these two reference genes is variably transcribed. In such event, ΔCT analysis was performed to compare each gene with all other genes and standard deviation of each gene was obtained. The reference gene with the lowest standard deviation was then selected as the most stable reference gene. Overall ranking of reference genes was confirmed by using the geometric mean of the rankings generated from the individual algorithms [23].

Analysis of gene expression using different reference genes for normalization

In order to evaluate the impact of using different reference gene for normalization on the expression levels measured by qRT-PCR, the two best-ranked, the two middle-ranked reference genes and a least-ranked reference genes were used to calculate the expression levels of hypothetical gene of interest along with the growth time for different serovars of *A*. *paragallinarum*. Expression levels of the hypothetical gene of interest were generated with normalization using the most stable reference genes, the stable reference genes and the least stable reference gene. The effect of reference genes with different ranks of stability was assessed by variation tendency of expression level for the hypothetical gene of interest along with the growth time for strains of *A*. *paragallinarum*.

Results

Primer amplification efficiency

The efficiency, linear dynamic range and specificity of nine pairs of primers were evaluated in qPCR with a series of five-fold dilution starting at 1:5 for a total of 5 dilutions. The efficiencies (E) of all primer pairs ranged from 88.2 to 112%, and correlation coefficients (R^2) were all higher than 0.98 (Table 1), both being considered acceptable [6,24]. Primer specificity was verified by the presence of a single-peak in the melting curve analysis in qRT-PCR (data not shown).

Expression profiles of candidate reference genes

In this study, certain variations in the expression levels of the nine candidate reference genes were observed in serovars A, serovars B and serovars C as shown in Fig 1A, Fig 1B and Fig 1C, respectively. The higher the level of gene expressed, the smaller the Cq value was. The Cq values of candidate genes ranged widely (10.48–36.24) in all tested samples. It was notable that the gene encoding superoxide dismutase (*sodA* gene) had the lowest expression levels in serovar A and C, reaching a cycle threshold after 35 amplification cycles and there was a significant difference in Cq value among serovars A, B, and C, while the mean Cq value of the *sodA* gene in serovar B was 24.27. The gene encoding 16S rRNA was highly expressed in all three serovars compared to the other genes, reaching a cycle threshold after only 10.48 amplification cycles.

GeNorm analysis

The expression stabilities of the nine candidate reference genes were analyzed using geNorm algorithms. High gene expression variability results in high M values and indicates low expression stability. For overall comparison, samples from three serovars at each growth phase (exponential and stationary) were calculated. In cultures of serovar A, *gyrA* (M = 0.468 and 0.428) was found to be most stably expressed gene while *sodA* (M = 1.781 and 1.257) was the least stably expressed both in exponential and stationary growth phase (Table 2). In cultures of serovar B, the most stable genes were found to be *atpD* (M = 0.438) and *recN* (M = 0.355) in exponential and stationary growth phase respectively, and the least stable gene was *sodA* (M = 1.046 and 0.686) (Table 3). In cultures of serovar C, *rpoB* (M = 0.347) and *recN* (M = 0.436) were found to be the most stably expressed genes in the exponential and stationary growth phase respectively while *sodA* (M = 1.154 and 1.026) was the least stably expressed both in the exponential and the stationary growth phase (Table 4). When combining exponential and the stationary phase, the most stable reference genes were 16S rRNA, *recN* and *gyrA*.

In addition to the ranking of the candidate reference genes, geNorm also recommends using optimal number of required reference genes and provides calculations of the impact of adding additional reference genes on normalization ($V_n/_{n+1}$). If a pairwise value ($V_n/_{n+1}$) is no more than 0.15, then there is no need to choose n+1 reference genes. In our study, the best combination of reference genes assessed by geNorm analysis was *gyrA* and *recN*, *atpD* and *gyrA*, *recN* and *rpoB* in exponential growth phase for serovars A, B, and C respectively (with $V_{2/3}$ = 0.053, 0.12, and 0.053, respectively). In stationary growth phase of serovars A, B, and C, however, the stability ranking-order seemed to vary with serovars. The optimal numbers and best combinations of reference genes in this phase were *gyrA* and *rpoB*, *gyrA* and *pgi*, *rpoB* and *atpD* for serovars A, B, and C respectively (with V2/3 = 0.052, 0.101, and 0.085, respectively). When combining the exponential and stationary growth phase, the optimal numbers and best combinations of reference genes were *recN* and 16S rRNA, *recN* and 16S rRNA, *recN*, *rpoB* and *gyrA* for serovars A, B, and C respecitively (with V2/3 = 0.086, V2/3 = 0.110, and V3/4 = 0.096, respectively).

NormFinder analysis

Stabilities of the expression of the nine reference genes were evaluated using NormFinder analysis. A lower stability value indicates a more stably expressed reference gene. NormFinder also suggests the best combination of two reference genes for quantitative real-time PCR normalization. For serovar A cultures, NormFinder identified *gyrA*, *atpD*, and *gyrA* as the most stable reference genes in exponential, stationary, and combined both growth phase, respectively (Table 5). For serovar B cultures, the most stable genes were found to be *atpD*, *recN*, and *gyrA*

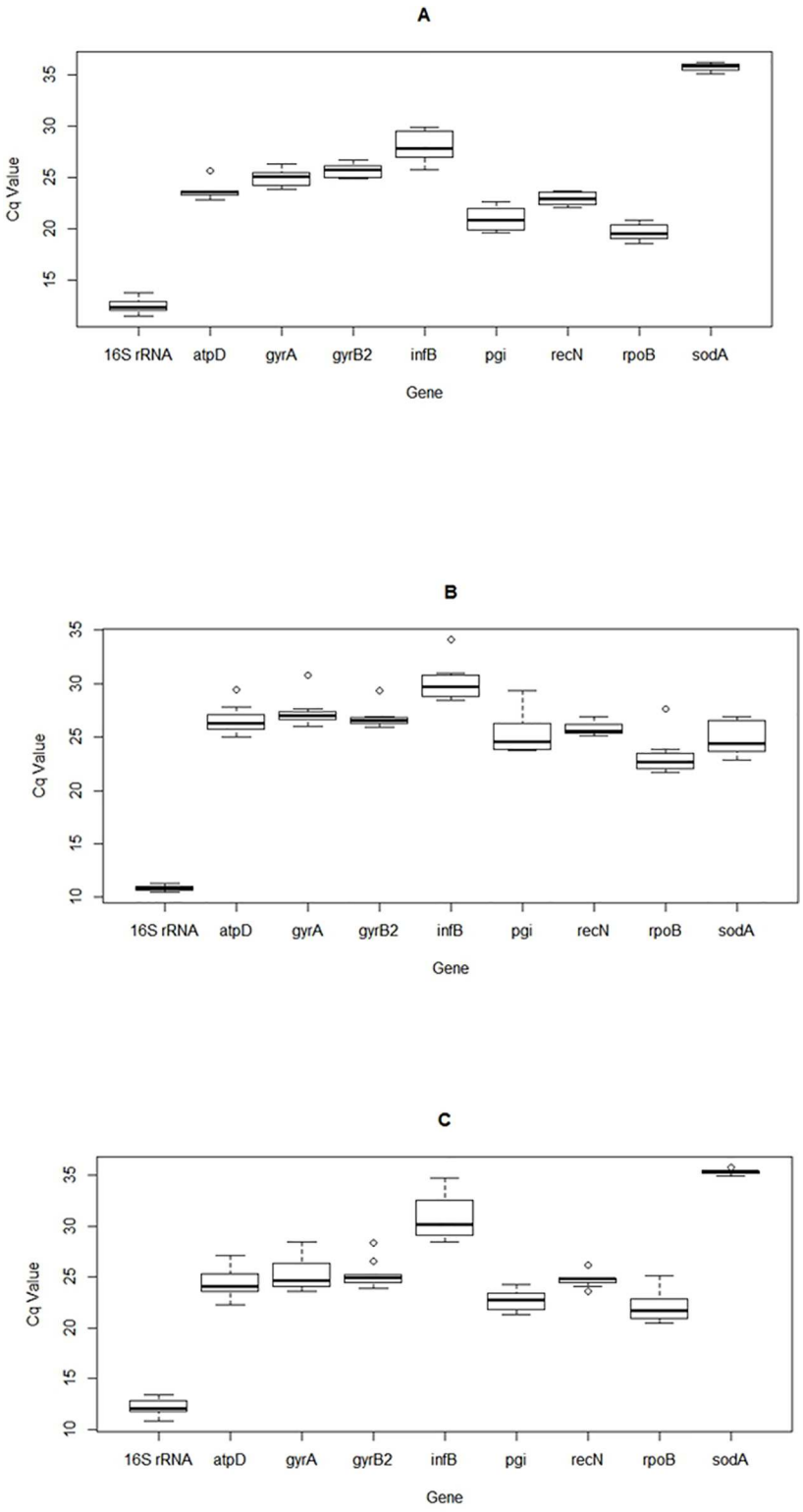

Fig 1. Range of Cq values of the nine candidate reference genes across all samples of *A. paragallinarum* serovars A, B, and C. Boxes and whiskers represent interquartile ranges and confidence intervals. Bars inside boxes indicate median values. Hollow circles show outliers (5th/95th percentile) respectively.

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Table 2. Gene expression stability rankings for different growth phase in *A. paragallinarum* serovar A analyzed by geNorm.

Exponential		Stationary		Combined	
gyrA	0.468	gyrA	0.428	16S rRNA	0.572
recN	0.480	atpD	0.436	gyrA	0.583
16S rRNA	0.513	rpoB	0.450	recN	0.590
rpoB	0.542	recN	0.470	rpoB	0.596
pgi	0.562	gyrB	0.484	pgi	0.604
atpD	0.606	infB	0.613	gyrB	0.666
gyrB	0.609	16S rRNA	0.625	atpD	0.673
infB	0.750	pgi	0.861	infB	1.009
sodA	1.781	sodA	1.257	sodA	2.040

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in the three growth phase respectively (Table 6). For serovar C cultures, the most stable genes were found to be *gyrA*, *gyrB*, and *gyrB* in the three growth phase respectively (Table 7).

ΔCT analysis

The comparative ΔCT method was used to assess the best reference gene by comparing standard deviation of a particular gene with all other genes. The results of the comparative ΔCT method analysis were similar to those from geNorm and Normfinder. However, both major and minor differences still exist in the tested samples from the other two analysis methods. A summary of the full results can be seen in Table 8.

Ranking of candidate reference genes

In order to mitigate potential biases introduced by any single calculation method, we developed a composite ranking based on geometric mean of the results from all three algorithms described above. The lower the geometric average is, the more stable of the candidate reference gene expresses. Here we briefly described our findings for different growth phase and different serovars. In serovar A cultures, the overall rankings are *gyrA*>*pgi*>*16S rRNA*>*recN*>*rpoB*>*gyrB*>*atpD*>*infB* in exponential phase, followed by *atpD*>*gyrA*>*rpoB*>*gyrB*>*recN*>*infB*>*16S rRNA*>*pgi* in stationary phase, then *gyrA*>*16S rRNA*>*recN*>*gyrB*>*rpoB*>*pgi*>*infB* in combined both growth phase (Table 9). In serovar B cultures, the overall rankings are *atpD*>*gyrA*>*16S rRNA*> *recN*>*pgi*>*gyrB*>*rpoB*>*infB*>*sodA* in exponential phase, followed by *recN*>*16S rRNA* > *pgi*>*gyrA*>*gyrB*>*rpoB*>*atpD*>*infB*>*sodA* in stationary phase, then *recN*>*16S rRNA*>*gyrA*>*gyrB*>*atpD*>*pgi*>*infB*>*rpoB*>*sodA* in combined both growth phase (Table 10). In serovar C

Table 3. Gene expression stability rankings for different growth phase in *A. paragallinarum* serovar B analyzed by geNorm.

Exponential		Stationary		Combined	
atpD	0.438	recN	0.355	recN	0.516
gyrA	0.447	16S rRNA	0.371	16S rRNA	0.554
recN	0.458	pgi	0.383	gyrA	0.556
16S rRNA	0.523	gyrA	0.384	atpD	0.593
pgi	0.530	rpoB	0.438	gyrB	0.644
gyrB	0.571	gyrB	0.476	infB	0.684
infB	0.602	atpD	0.488	pgi	0.711
rpoB	0.673	infB	0.600	rpoB	0.801
sodA	1.046	sodA	0.686	sodA	1.539

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Table 4. Gene expression stability rankings for different growth phase in *A. paragallinarum* serovar C analyzed by geNorm.

Exponential		Stationary		Combined	
rpoB	0.347	recN	0.436	gyrA	0.502
recN	0.365	gyrB	0.440	recN	0.502
gyrA	0.378	rpoB	0.506	gyrB	0.504
gyrB	0.395	gyrA	0.515	rpoB	0.536
16S rRNA	0.429	pgi	0.519	16S rRNA	0.580
pgi	0.471	atpD	0.575	atpD	0.593
infB	0.542	16S rRNA	0.584	pgi	0.617
atpD	0.561	infB	0.825	infB	0.747
sodA	1.154	sodA	1.026	sodA	0.877

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cultures, the overall rankings are *rpoB*>*recN*>*gyrA*>*gyrB*>*16S rRNA*>*pgi*>*infB*>*atpD* in exponential phase, followed by *recN*>*gyrB*>*rpoB*>*gyrA*>*pgi*>*atpD*>*16S rRNA*>*infB* in stationary phase, then *gyrB*> *gyrA*> *recN*> *rpoB*>*16S rRNA*> *atpD*> *pgi*>*infB* in combined both growth phase (Table 11).

Impact of reference gene selection on gene expression studies

To determine the effect of a poorly ranked reference gene on a gene expression study, we performed a 50S ribosomal protein L33 expression analysis using data from the cultures of serovar B of *A. paragallinarum* in stationary growth phase. Expression of 50S ribosomal protein L33 was assessed using two highly ranked genes: *recN*, *16S rRNA*, two middle-ranked reference genes: *gyrA* and *atpD* and a poorly ranked gene-*sodA*. The results revealed a difference in tendency of expression level for 50S ribosomal protein L33 along with the growth time. There was a same tendency when normalised to the two most stable reference genes and two stable reference genes, as opposed to inconsistent result when normalised to the least stable reference gene (Fig 2). Therefore, by using the least stable reference gene *sodA* for normalization significantly changed the calculated expression level of 50S ribosomal protein L33 which could lead to large error alterations in study results.

Discussion

Real-Time quantitative PCR is among the most powerful tools for detection of expression levels of target genes. Endogenous reference genes are widely used in qPCR assays for normalization because their stability in different experimental conditions and biological treatment

Table 5. Gene expression stability rankings for different growth phase in *A. paragallinarum* serovar A analyzed by NormFinder.

Exponential		Stationary		Combined	
gyrA	0.028	atpD	0.036	gyrA	0.047
recN	0.028	gyrB	0.039	16S rRNA	0.062
16S rRNA	0.056	gyrA	0.042	recN	0.062
gyrB	0.063	rpoB	0.042	pgi	0.084
pgi	0.170	recN	0.120	gyrB	0.091
rpoB	0.206	infB	0.275	rpoB	0.157
atpD	0.333	16S rRNA	0.343	atpD	0.324
infB	0.483	pgi	0.568	infB	0.663
sodA	1.229	sodA	0.853	sodA	1.408

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Table 6. Gene expression stability rankings for different growth phase in *A. paragallinarum* serovar B analyzed by NormFinder.

exponential		stationary		Combined	
atpD	0.030	recN	0.016	gyrA	0.042
gyrA	0.050	16S rRNA	0.081	recN	0.043
recN	0.134	pgi	0.136	16S rRNA	0.124
pgi	0.204	gyrA	0.137	atpD	0.198
16S rRNA	0.263	rpoB	0.227	pgi	0.252
infB	0.283	gyrB	0.253	gyrB	0.274
gyrB	0.285	atpD	0.273	infB	0.331
rpoB	0.420	infB	0.391	rpoB	0.496
sodA	0.705	sodA	0.458	sodA	1.051

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system [18,25].Housekeeping genes, such as *18S* and *28S rRNA*, *GAPD (3- GAPDH)*, *ACTB (actin)*, and *TUBLIN (tubulin)*, etc., are often used as reference genes in relative quantification as the proteins encoded s are essential for maintaining cellular activities and are stably expressed in different tissues and organs [25,26]. However, the expression levels of reference genes are only conditionally stable and are subject to different species, experimental conditions, growth phase, biotic and abiotic stimulations. Each particular experimental condition, therefore, has its suitable stable expression reference genes [27–29] and none of the commonly used reference gene is universal [30–32]. For example, four genes, *rpoB*, *atpD*, *gyrA* and *gyrB*, were found to be most stable candidate reference genes; whereas the expression of *16S rRNA*, a commonly used reference gene in many of studies, has been found to be unstable [33]. Furthermore, the expression of some genes varies depending on different growth conditions and stimulations. One study found that *pyk* and *rpoB* performed most stably when comparing aerobic and epinephrine cultures during growth phase; whereas when analyzing exponential and stationary growth phase together, only *pyk* remained in the top three rankings and the *16S rRNA* has been demonstrated unstable under certain study conditions [14]. Choosing a suitable reference gene for gene expression research based on experimental conditions is critical for valid analysis.

In addition, the *atpD* gene we identified is a highly conservative and stable gene in *Pasteurellaceae*, and is commonly used as a molecular biomarker [9] for bacterial categorization and widely used as an reference gene in regenerating phylogenetic tree for *Pasteurellaceae* in recent studies [34]. However, current study is the first to demonstrate its existence in *A. paragallinarum*. To provide reference for future studies, we sequenced *A. paragallinarum atpD* gene of serovar A, B and C strains and deposited those into GenBank (KXO78457, KXO78458 and KXO78459).

Table 7. Gene expression stability rankings for different growth phase in *A. paragallinarum* serovar C analyzed by NormFinder.

exponential		stationary		Combined	
gyrA	0.007	gyrB	0.056	gyrB	0.152
recN	0.029	recN	0.056	gyrA	0.154
rpoB	0.029	gyrA	0.142	recN	0.161
gyrB	0.055	pgi	0.172	rpoB	0.229
16S rRNA	0.134	rpoB	0.219	16S rRNA	0.259
pgi	0.227	16S rRNA	0.273	atpD	0.294
infB	0.304	atpD	0.311	pgi	0.323
atpD	0.338	infB	0.553	infB	0.460
sodA	0.788	sodA	0.699	sodA	0.553

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Table 8. Gene expression stability assessed by the comparative ΔCT method.

Serovar A						Serovar B						Serovar C					
Exponential		Stationary		Combined		Exponential		Stationary		Combined		Exponential		Stationary		Combined	
gene	Mean s.d	gene	Mean s.d	gene	Mean s.d	gene	Mean s.d	gene	Mean s.d	gene	Mean s.d	gene	Mean s.d	gene	Mean s.d	gene	Mean s.d
pgi	0.32	atpD	0.32	gyrB	1.70	16S rRNA	0.41	recN	0.36	16S rRNA	0.24	rpoB	0.24	recN	0.35	gyrB	0.44
rpoB	0.34	gyrA	0.33	recN	2.36	atpD	0.44	pgi	0.38	recN	0.31	recN	0.26	gyrB	0.38	recN	0.56
16S rRNA	0.36	recN	0.35	gyrA	2.45	gyrA	0.45	gyrA	0.38	gyrB	0.35	gyrA	0.28	rpoB	0.40	rpoB	0.62
gyrA	0.39	rpoB	0.35	rpoB	2.50	recN	0.46	16S rRNA	0.44	gyrA	0.41	gyrB	0.32	gyrA	0.46	gyrA	0.63
gyrB	0.40	gyrB	0.41	16S rRNA	2.50	rpoB	0.50	gyrB	0.48	rpoB	0.56	16S rRNA	0.34	atpD	0.47	16S rRNA	0.63
atpD	0.43	16S rRNA	0.54	atpD	2.74	pgi	0.53	atpD	0.49	atpD	0.56	pgi	0.36	pgi	0.49	pgi	0.69
recN	0.45	infB	0.56	pgi	2.81	gyrB	0.57	infB	0.60	infB	0.65	infB	0.43	16S rRNA	0.59	atpD	0.77
infB	0.73	pgi	0.73	infB	2.91	infB	0.60	rpoB	0.61	pgi	0.70	atpD	0.44	infB	0.71	infB	0.92
						sodA	1.05	sodA	0.69	sodA	1.63						

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In this study, no universal reference gene was found for all environments and phase. This was probably contributed by variations among different serovars, i.e. the stable reference genes in one serovar might not necessarily be stable in another serovar. Then, the expression level of the same reference gene might vary at different growth phase, such as exponential and stationary phase. Therefore, when choosing reference gene for normalization of expression levels, the timing and serovars need to be taken into consideration for most accurate estimation of expression levels.

The *gyrA* and *rpoB*, which have been evaluated in *Actinobacillus* [14], *Staphylococcus aureus* [35], and *Xanthomona s* [33], and demonstrated most stable expression characteristics, also exhibited stability in our study. *16S rRNA*, a widely used reference gene for species classification, had a low favorably ranking despite its high-abundance with mRNA transcription. Interestingly, compared to other reference genes, *sodA*, which was usually used for strain identification

Table 9. Candidate reference genes ranked by different methods in serovar A.

Exponential					Stationary					Combined				
geN	Norm	ΔCT	Ranking	Mean	geN	Norm	ΔCT	Ranking	Mean	geN	Norm	ΔCT	Ranking	Mean
gyrA	gyrA	pgi	gyrA	1.59	gyrA	atpD	atpD	atpD	1.26	16S rRNA	gyrA	gyrB	gyrA	1.82
recN	recN	rpoB	pgi	2.92	atpD	gyrB	gyrA	gyrA	1.82	gyrA	16S rRNA	recN	16S rRNA	2.15
16S rRNA	16S rRNA	16S rRNA	16S rRNA	3.00	rpoB	gyrA	rpoB	rpoB	3.30	recN	recN	gyrA	recN	2.62
rpoB	gyrB	gyrA	recN	3.04	recN	rpoB	recN	gyrB	3.42	rpoB	pgi	rpoB	gyrB	3.11
pgi	pgi	gyrB	rpoB	3.63	gyrB	recN	gyrB	recN	3.91	pgi	gyrB	16S rRNA	rpoB	4.58
atpD	rpoB	atpD	gyrB	5.19	infB	infB	16S rRNA	infB	6.00	gyrB	rpoB	atpD	pgi	5.19
gyrB	atpD	recN	atpD	6.32	16S rRNA	16S rRNA	infB	16S rRNA	6.26	atpD	atpD	pgi	atpD	6.65
infB	infB	infB	infB	8.00	pgi	pgi	pgi	pgi	7.65	infB	infB	infB	infB	8.00

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Table 10. Candidate reference genes ranked by different methods in serovar B.

Exponential					Stationary					Combined				
geN	Norm	ΔCT	Ranking	Mean	geN	Norm	ΔCT	Ranking	Mean	geN	Norm	ΔCT	Ranking	Mean
atpD	atpD	16S rRNA	atpD	1.26	recN	recN	recN	recN	1.00	recN	gyrA	16S rRNA	recN	1.59
gyrA	gyrA	atpD	gyrA	2.29	16S rRNA	16S rRNA	pgi	16S rRNA	2.29	16S rRNA	recN	recN	16S rRNA	1.82
recN	recN	gyrA	16S rRNA	2.71	pgi	pgi	gyrA	pgi	2.62	gyrA	16S rRNA	gyrB	gyrA	2.29
16S rRNA	pgi	recN	recN	3.30	gyrA	gyrA	16S rRNA	gyrA	3.17	atpD	atpD	gyrA	gyrB	4.48
pgi	16S rRNA	rpoB	pgi	4.93	rpoB	rpoB	gyrB	gyrB	5.24	gyrB	pgi	rpoB	atpD	4.58
gyrB	infB	pgi	gyrB	6.65	gyrB	gyrB	atpD	rpoB	5.59	infB	gyrB	atpD	pgi	6.54
infB	gyrB	gyrB	rpoB	6.84	atpD	atpD	infB	atpD	6.26	pgi	infB	infB	infB	6.65
rpoB	rpoB	infB	infB	6.95	infB	infB	rpoB	infB	7.27	rpoB	rpoB	pgi	rpoB	6.84
sodA	sodA	sodA	sodA	9.00	sodA	sodA	sodA	sodA	8.65	sodA	sodA	sodA	sodA	9.00

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and species classification [10,35], had a high expression level in serovar B, but not in A and C (with CT = 35), and amazingly kept CT values unchanged regardless of assay conditions. This meant that expression levels of *sodA* was very low in serovar A and C beyond the limit of detection for the quantitative PCR, *sodA* was therefore excluded from the ΔCT analysis. In addition, the stabililty ranking of *sodA* was at the bottom for serovar B, which further confirmed that *sodA* was not suitable for normalization in qPCR assays of *A*. *paragallinarum*. The stable ranking of reference genes analyzed by geNorm was generally consistent with that by NormFinder, with minor differences in individual reference genes. For example, the top four stable reference genes analyzed by geNorm were *gyrA*, *atpD*, *rpoB*, and *recN*; while NormFinder recommended the use of *atpD*、*gyrB*、*gyrA*, and *rpoB* for normalization. The variations may be explained by different parameter settings, assumptions and algorithms in geNorm and NormFinder when calculating the gene expression stability of the reference genes. Thus, a combination of two or more software was needed for the stability ranking of candidate genes when selecting reliable reference genes [36].

In conclusion, suitable reference gene candidates were selected for use in serovars A, B, and C of *A*. *paragallinarum* in different growth phase. For serovar A, *gyrA* and *atpD* were the most

Table 11. Candidate reference genes ranked by different methods in serovar C.

Exponential					Stationary					Combined				
geN	Norm	ΔCT	Ranking	Mean	geN	Norm	ΔCT	Ranking	Mean	geN	Norm	ΔCT	Ranking	Mean
rpoB	gyrA	rpoB	rpoB	1.44	recN	gyrB	recN	recN	1.26	gyrA	gyrB	gyrB	gyrB	1.44
recN	recN	recN	recN	2.00	gyrB	recN	gyrB	gyrB	1.59	recN	gyrA	recN	gyrA	2.00
gyrA	rpoB	gyrA	gyrA	2.08	rpoB	gyrA	rpoB	rpoB	3.56	gyrB	recN	rpoB	recN	2.29
gyrB	gyrB	gyrB	gyrB	4.00	gyrA	pgi	gyrA	gyrA	3.63	rpoB	rpoB	gyrA	rpoB	3.63
16S rRNA	16S rRNA	16S rRNA	16S rRNA	5.00	pgi	rpoB	atpD	pgi	4.93	16S rRNA	16S rRNA	16S rRNA	16S rRNA	4.64
pgi	pgi	pgi	pgi	6.00	atpD	16S rRNA	pgi	atpD	5.94	atpD	atpD	pgi	atpD	6.00
infB	infB	infB	infB	7.00	16S rRNA	atpD	16S rRNA	16S rRNA	6.65	pgi	pgi	atpD	pgi	6.26
atpD	atpD	atpD	atpD	8.00	infB	infB	infB	infB	8.00	infB	infB	infB	infB	7.65

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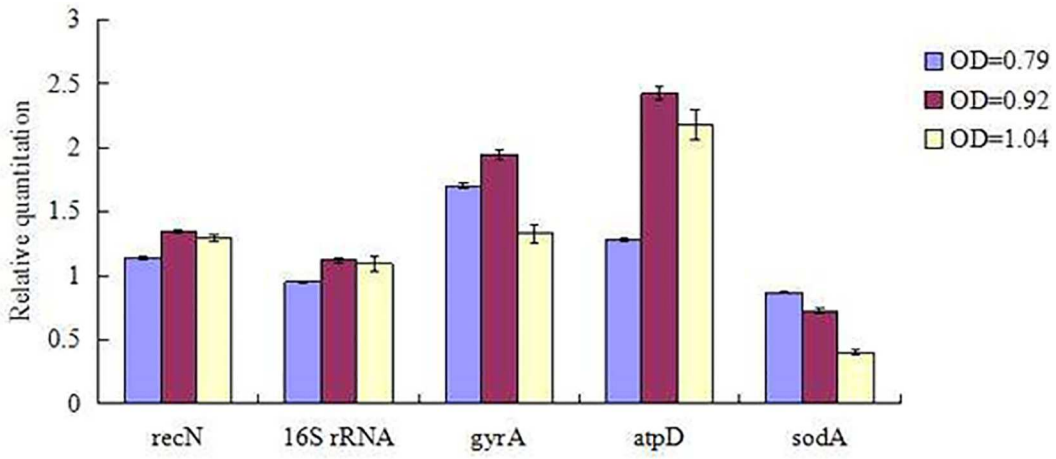

Fig 2. 50S ribosomal protein L33 expression analysis at different time points in stationary phase in cultures of serovar B of *A. paragallinarum*. Different colored columns show the relative quantification of 50S ribosomal protein L33 when normalized against reference genes at three sampling time points. *RecN* and *16S rRNA* were the two highest ranked genes, *gyrA* and *atpD* were the two highly ranked genes for cultures of serovar B of *A. paragallinarum* in stationary phase and *sodA* was a poorly ranked gene.

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stably expressed in exponential and stationary phase respectively; for serovar B, *atpD* and *recN* were the most stably expressed in exponential and stationary phase respectively; for serovar C, *rpoB* and *recN* were the most stably expressed in exponential and stationary phase respectively.

Author Contributions

Conceptualization: HS.

Data curation: SW.

Formal analysis: SW.

Funding acquisition: HS.

Investigation: SW.

Methodology: HS.

Project administration: HS.

Resources: HS.

Supervision: HS XC FX.

Validation: SW.

Visualization: HS SW.

Writing – original draft: SW.

Writing – review & editing: HS XC FX.

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