Bootstrap confidence intervals for nucleic acid concentration in absolute real-time PCR quantification

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Summary

Objectives. The aim of this study was to evaluate, in the context of absolute real-time polymerase chain reaction (PCR) analysis, four bootstrap methods of computing confidence intervals for the target nucleic acid concentration.

Methods. Starting from real data, 500 Monte Carlo simulations were implemented under the assumption of identically and independently normally distributed errors for two specified values of standard deviation and for four fixed true concentration values. For each data set 95% confidence intervals of the true concentration were computed by means of the Delta method and by applying four different bootstrap methods: standard bootstrap, percentile bootstrap, bias corrected bootstrap and bootstrap-t.

Results. Both the Delta method and the bootstrap-t method provided confidence intervals with acceptable coverage probability for the whole set of tested cases. On the contrary, the other bootstrap methods gave confidence intervals falling outside the coverage acceptance range in ten cases. In all cases the median and the inter-quartile range of the width distributions of the confidence intervals for the Delta and bootstrap-t methods were similar and slightly greater than for the remaining ones.

Conclusions. The results showed that the performances of the Delta method and the bootstrap-t are comparable in the framework of absolute real-time PCR quantification.

KEY WORDS: *absolute real-time PCR, bootstrap methods, confidence intervals, coverage probability, Monte Carlo simulation*.

Introduction

The real-time polymerase chain reaction (PCR) is a molecular technique widely used for the quantification of nucleic acids in a broad range of clinical and research applications including the measurement of gene dosage, detection of residual disease in haematological malignancies and detection of bacterial and viral infection (1). The real-time PCR typically employs fluorescent probes which generate a signal that accumulates during PCR cycling in a manner proportional to the concentration of amplification products. In real-time PCR data analysis, the cycle threshold (c_t) method is currently the gold standard and, even though we are aware that alternative methods have been suggested (2), the c_t is the method we will focus on in this study, as it is based on an assumption of equal PCR efficiency in all reactions.

Absolute quantification of target nucleic acids can be achieved using a standard curve, which is generated by amplifying known amounts of nucleic acid. To construct the standard curve a set of 10-fold dilutions of a positive control template is used as standard. By means of a technique known as "inverse regression", the standard curve is used as a "calibrator" to estimate the unknown nucleic acid concentration in the sample under examination. As in any titration, evaluating the uncertainty in the estimated concentration

is critical for interpreting the data and optimizing the experimental procedures.

In a previous paper (3) two of us (PV and EM) advocated the use of Fieller's theorem (4) to estimate the confidence interval for nucleic acid concentration by real-time PCR. In addition to the Fieller's theorem, the Delta method (5) is also commonly used, in biochemical applications, to compute confidence intervals in inverse calibrations. More recently, in a tutorial paper (6), the relationship between these two methods was debated and their geometrical interpretation was shown. The paper concluded that, from a practical viewpoint, in real-time PCR quantification the two methods give overlapping results.

Alternatively, some authors [for example Bonate (7) and Jones and Rocke (8)] suggested using the bootstrapping approach to provide confidence intervals in inverse calibration settings.

In the context of absolute real-time PCR quantification the aim of the present paper was to evaluate the performance, in computing confidence intervals, of four bootstrap methods by comparing their coverage and width with the coverage and width provided by the Delta method. Starting from real data this was done using Monte Carlo simulations under the assumption of identically and independently normally distributed errors.

Material and Methods

Background models

The basic equation describing real-time PCR kinetics is:

$$N_c = N_0 E^c \quad [1]$$

where N_c is the template concentration at cycle c, N_0 is the starting template concentration and E is the amplification efficiency. The latter can be thought of as the yield of the amplification reaction and two is its ideal value, corresponding to a yield of 100%: in this case every molecule is duplicated at each cycle so that the template concentration at cycle c would be twice the template concentration at cycle c – 1.

By taking the common logarithm, equation 1 becomes:

$$Log(N_c) = Log(N_0) + c[Log(E)] \quad [2]$$

To establish a direct connection between the above relation and the standard dilutions it is useful to rewrite equation 2 as:

$$c_{ts} = \frac{Log(N_{c_t})}{Log(E)} - \frac{1}{Log(E)} Log(N_{0_s}) \quad [3]$$

where the subscripts t and s mean threshold and standard dilution, respectively, so that c_{ts} identifies, for each standard dilution, the fractional cycle where a threshold amount of amplified nucleic acid (N_{c_t}) is produced.

Equation 3 describes the linear relationship between the c_{ts} values (dependent variable) and the logarithm of the known starting concentration of the standard dilutions (independent variable), with the intercept $\beta_0 = \frac{Log(N_{c_t})}{Log(E)}$ and the slope $\beta_1 = -\frac{1}{Log(E)}$. The straight line in equation 3 represents the so-called standard curve, used as calibrator to estimate the unknown nucleic acid concentrations of the different samples to be tested.

The statistical model corresponding to the standard curve is:

$$y_{ij} = \beta_0 + \beta_1 x_i + \varepsilon_{ij} \quad [4]$$

where y_{ij} specifies the value of c_t measured for the j-th replication (j=1,2,…,J_i) at the i-th standard dilution (i=1,2,…,I), x_i defines the logarithm of the starting nucleic acid concentration of the i-th standard dilution and ε_{ij} is the random component assumed to be identically and independently normally distributed with mean zero and constant error variance σ^2. The estimates b_0 and b_1 of β_0 and β_1 respectively are obtained by the ordinary least squared method (OLS).

The value of interest in a real-time PCR experiment is the logarithm of the unknown nucleic acid concentration in the sample under investigation. The latter (x_0) is usually estimated by resorting to the following inverse regression:

$$\hat{x}_0 = \frac{\bar{y}_0 - b_0}{b_1} \quad [5]$$

where $\bar{y}_0$ is the mean of K replicated values of c_t [i.e. $c_{tk} = y_k$, (k=1,2,…K)] measured for the sample under investigation.

Delta method

The Delta method provides an approximate variance of the ratio of random variables based on a first-order Taylor series expansion (5). Thus, the variance of $\hat{x}_0$ is estimated as:

$$s^2(\hat{x}_0) \approx \left(\frac{s^2}{b_1^2}\right)\left(\frac{(\hat{x}_0 - \bar{\bar{x}})^2}{s_{xx}} + \frac{1}{K} + \frac{1}{n}\right) \quad [6]$$

where $s^2 = \dfrac{\sum_{i=1}^{I}\sum_{j=1}^{J_i}(y_{ij} - \hat{y}_i)^2}{f}$ is the estimated error variance of the model in equation 4 with $f = (I-2) + \sum_{i=1}^{I}(J_i - 1)$ degrees of freedom, $n = \sum_{i=1}^{I} J_i$, s_{xx} and $\bar{\bar{x}}$ are the sum of squares and the mean of the x_i values, respectively. The limits of the 100 $(1 - \alpha)$% confidence interval of x_0 can be written as:

$${}_D\hat{x}_L = \hat{x}_0 - t_{f;1-\alpha/2}\,s(\hat{x}_0), \qquad {}_D\hat{x}_U = \hat{x}_0 + t_{f;1-\alpha/2}\,s(\hat{x}_0). \quad [7]$$

where, $t_{f;1-\alpha/2}$ is the critical value corresponding to a prefixed $100(1 - \alpha/2)$% level of the Student's t-distribution with f degrees of freedom.

Simulation plan

The structure of the Monte Carlo simulation scheme mimicked the design adopted in a European External Quality Assessment (EQA) programme for quantitative real-time PCR assays (9,10). Briefly, the standard curve was based on five standard dilutions (I=5) containing 10, 10^2, 10^3, 10^4, 10^5 copies/5 µL, respectively. Three replicates of c_t were assayed for each standard dilution (J_i=3, for i=1,2,…,5) as well as for the unknown concentration sample (K=3).

The simulation starting value for β_1 was -3.637 corresponding to the median value of the b_1 distribution obtained from the data of the participants in the abovementioned EQA programme. The starting value for β_0 was 43.814 pertaining to the laboratory whose b_1 was the median value. Note that the efficiency corresponding to β_1 is E=1.88, slightly lower than the ideal value.

Following the linear regression model in equation 4, M independent simulated data sets were obtained by considering two different scenarios for generating random errors (ε_{ij}): in the first the values of ε_{ij} were generated from a normal distribution with µ=0 and σ=0.2, whereas in the second the values of ε_{ij} were generated from a normal distribution with µ=0 and σ=0.7. These two σ values corresponded approximately to the 10th and 90th centiles respectively of the distribution of the estimated standard deviation of the EQA participants.

As regards the unknown sample, four different values of the true concentration x_0 were fixed as follows: x_1=1.5, x_2=2.5, x_3=3.5 and x_4=4.5. These concentrations correspond to the middle points between two consecutive standard dilutions.

Owing to the structure of the design, for each value of x_0 a specific data set of 18 observations was simulated. First, 18 random numbers were generated, 15 for the five standard dilutions (ε_{ij}) and three for the unknown sample (ε_k), from each of the two normal distributions considered. Then the simulated c_t values (y_{ij} or y_{0k}) were computed as follows: $y_{ij} = \beta_0 + \beta_1 x_i + \varepsilon_{ij}$ for the standard dilutions and $y_{0k} = \beta_0 + \beta_1 x_0 + \varepsilon_k$ for the unknown sample considered. Random numbers were obtained using the RANNOR function of the SAS® package (11). The computer clock was used to specify seed values. In detail, by using different starting seeds for the eight different scenarios and the same seed within each scenario M=500 data sets were simulated for each scenario. In the worst case (x_0 =1.5) the level of accuracy (12) of the x_0 estimate corresponding to 500 simulations ranges between 0.2% and 0.8%, according to the two values of σ considered, namely σ=0.2 and σ=0.7.

For each data set the standard curve was built and the corresponding parameters (b_0 and b_1) were estimated by the OLS. Using equations 5 and 7, the unknown concentration estimate ($\hat{x}_0$) and the $100(1 - \alpha)$% confidence interval of x_0 were computed.

Bootstrap resampling plan

Starting from each fitted standard curve 15 residuals ($r_{ij} = y_{ij} - \hat{y}_i$) were gathered and after each of them had been multiplied by the adjustment factor $\sqrt{n/(n-h)}$ the corresponding $\tilde{r}_{ij}$ residuals were obtained, where h is the number of parameters (13). Furthermore, for the unknown sample, after comput-

ing the three residuals $r_k = y_{0k} - \bar{y}_0$ the corresponding $\tilde{r}_k$ were obtained by multiplying the r_k residuals by the adjustment factor $\sqrt{3/(3-1)}$. The whole set of 18 residuals became the components of the residuals pool for the bootstrap resampling. Sampling with replacements from this pool gives the eighteen bootstrap residuals ($\tilde{r}_{ij}^*$ and $\tilde{r}_k^*$) for the computation of the corresponding bootstrap data: $y_{ij}^* = b_0 + b_1 x_i + \tilde{r}_{ij}^*$ for the standard dilutions and $y_{0k}^* = \bar{y}_0 + \tilde{r}_k^*$ for the unknown sample. The bootstrap standard curve parameters (b_0^* and b_1^*) were estimated by the OLS. The bootstrap estimate of x_0 was computed as follows:

$$\hat{x}_0^* = \frac{\bar{y}_0^* - b_0^*}{b_1^*}, \quad \text{where} \quad \bar{y}_0^* = \frac{\sum_{k=1}^{K} y_{0k}^*}{K}.$$

The bootstrap resampling number was B=999 for each simulation (14).

Bootstrap confidence intervals

Four methods were considered for the computation of bootstrap confidence intervals of x_0: standard bootstrap, percentile bootstrap, bias-corrected percentile bootstrap and bootstrap-t.

The first method is based on the bootstrap standard deviation of $\hat{x}_0^*$ computed as:

$$s(\hat{x}_0^*) = \left\{ \frac{\sum_{b=1}^{B} [\hat{x}_0^*(b) - \hat{x}_0^*(.)]^2}{B-1} \right\}^{1/2}$$

where $\hat{x}_0^*(.) = \frac{\sum_{b=1}^{B} \hat{x}_0^*(b)}{B}$, so that the limits of the $100(1-\alpha)\%$ standard bootstrap confidence interval were: ${}_{SB}\hat{x}_L^* = \hat{x}_0 - z_{1-\alpha/2} s(\hat{x}_0^*)$ and ${}_{SB}\hat{x}_U^* = \hat{x}_0 + z_{1-\alpha/2} s(\hat{x}_0^*)$, where $z_{1-\alpha/2}$ is the $(1-\alpha/2)$ centile of the standard normal distribution.

The limits of the $100(1-\alpha/2)\%$ percentile bootstrap confidence interval (${}_{PB}\hat{x}_L^*$ and ${}_{PB}\hat{x}_U^*$) were just the $100(\alpha/2)^{th}$ and $100(1-\alpha/2)^{th}$ centile of the distribution of the 999 $\hat{x}_0^*$ values.

The computation of the bias-corrected confidence intervals implied:

– counting the number of times (d) that $\hat{x}_0^* < \hat{x}_0$;

– setting $\hat{z}_0 = \phi^{-1}(d/B)$, where $\phi^{-1}(.)$ indicates the inverse of the cumulative distribution function of the standard normal distribution;

– computing $\alpha_1 = \phi(2\hat{z}_0 + z_{\alpha/2})$ and $\alpha_2 = \phi(2\hat{z}_0 + z_{1-\alpha/2})$. The $100\alpha_1^{th}$ and $100\alpha_2^{th}$ centiles of the $\hat{x}_0^*$ distribution were the bias-corrected bootstrap confidence limits of x_0 (${}_{BC}\hat{x}_L^*$ and ${}_{BC}\hat{x}_U^*$).

For the computation of the bootstrap-t confidence interval the following pivotal statistic was computed for each data set:

$$t^* = \frac{\hat{x}_0^* - \hat{x}_0}{se(\hat{x}_0^*)}$$

where, se ($\hat{x}_0^*$) was calculated as the square root of the approximate variance of $\hat{x}_0$ (equation 6) using the values obtained from each bootstrap dataset. From the distribution of the t^* values the $100(\alpha/2)^{th}$ and $100(1-\alpha/2)^{th}$ centiles were found and the lower and upper limits of the bootstrap-t confidence intervals were:

$${}_{BT}\hat{x}_L^* = \hat{x}_0 - t_{1-\alpha/2}^* s(\hat{x}_0) \quad \text{and} \quad {}_{BT}\hat{x}_U^* = \hat{x}_0 - t_{\alpha/2}^* s(\hat{x}_0).$$

Coverage assessment

The estimated coverage probability is the proportion of M confidence intervals containing the true concentration value. The coverage should be approximately equal to the nominal coverage. A possible criterion for acceptability of the coverage is that the estimated coverage should not fall outside approximately two times the standard error (SE) of the nominal coverage probability (p), $SE(p) = \sqrt{p(1-p)/M}$ (12).

Software

A specific code was developed in SAS® package (11) to carry out data analysis.

Results

Table 1 reports the estimates of the coverage probability of the 95% confidence intervals for whole set of adopted methods, for the two values of σ and for the four values of x_0: in total 40 cases. The symbol (°) identified the cases in which the coverage was unsat-

Table1. Coverage probability estimates.

	x_0 / Method	1.5	2.5	3.5	4.5
σ = 0.2	Delta	95.6	94.6	95.2	95.4
	SB	94.8	91.6 (°)	92.8 (°)	94.0
	PB	95.2	91.8 (°)	93.0 (°)	94.2
	BC	95.2	91.6 (°)	93.0 (°)	94.2
	BT	95.4	93.8	94.8	95.2
σ = 0.7	Delta	94.0	94.8	96.0	94.2
	SB	94.2	93.2	94.8	92.6 (°)
	PB	93.4	93.4	94.2	92.4 (°)
	BC	93.0 (°)	93.2	94.2	92.4 (°)
	BT	94.6	94.8	95.6	95.0

(°)=estimated coverage probability outside the adopted range of acceptance (93.1%, 96.9%).
x_0=true concentration value.
SB=standard bootstrap.
PB=percentile bootstrap.
BC=bias-corrected percentile bootstrap.
BT=bootstrap-t.

isfactory according to the adopted range of acceptance (93.1% and 96.9%). Both the Delta method and the bootstrap-t provided confidence intervals with acceptable coverage for the whole set of tested cases. Furthermore, the coverage of the two methods was similar and very close to the nominal one.
On the contrary the remaining bootstrap methods gave confidence intervals falling outside the range of acceptance in ten cases (Table 1).
Table 2 reports descriptive statistics for interval width distributions. In all cases the medians and the inter-quartile ranges of the Delta method and the bootstrap-t were very similar and slightly greater than those of the remaining methods.

Table 2. Interval width distributions: descriptive statistics.

σ	x_0	method	min	median	max	IQR
0.2	1.5	Delta	0.0695	0.1599	0.2840	0.0435
		SB	0.0743	0.1463	0.2340	0.0384
		PB	0.0734	0.1460	0.2413	0.0387
		BC	0.0727	0.1452	0.2404	0.0387
		BT	0.0709	0.1597	0.2769	0.0469
	2.5	Delta	0.0695	0.1521	0.2766	0.0433
		SB	0.0638	0.1392	0.2285	0.0344
		PB	0.0632	0.1390	0.2253	0.0344
		BC	0.0638	0.1396	0.2240	0.0348
		BT	0.0664	0.1507	0.2771	0.0430
	3.5	Delta	0.0693	0.1471	0.2361	0.0379
		SB	0.0690	0.1340	0.2023	0.0329
		PB	0.0706	0.1336	0.1981	0.0324
		BC	0.0708	0.1344	0.2031	0.0330
		BT	0.0731	0.1462	0.2513	0.0384
	4.5	Delta	0.0716	0.1591	0.2685	0.0453
		SB	0.0726	0.1446	0.2300	0.0390
		PB	0.0730	0.1455	0.2339	0.0388
		BC	0.0721	0.1445	0.2323	0.0383
		BT	0.0698	0.1587	0.2739	0.0455

Table 2.

σ	x_0	method	min	median	max	IQR
0.7	1.5	Delta	0.2735	0.5529	0.9380	0.1546
		SB	0.2643	0.5062	0.8036	0.1244
		PB	0.2602	0.5104	0.8406	0.1273
		BC	0.2599	0.5085	0.8348	0.1265
		BT	0.2733	0.5479	0.9571	0.1595
	2.5	Delta	0.2729	0.5251	0.9009	0.1366
		SB	0.2371	0.4763	0.7952	0.1236
		PB	0.2361	0.4780	0.7903	0.1188
		BC	0.2341	0.4785	0.7876	0.1213
		BT	0.2618	0.5162	0.9579	0.1397
3.5		Delta	0.2496	0.5160	0.8416	0.1528
		SB	0.2539	0.4736	0.7711	0.1303
		PB	0.2476	0.4706	0.7567	0.1334
		BC	0.2472	0.4683	0.7543	0.1314
		BT	0.2462	0.5111	0.8961	0.1507
4.5		Delta	0.2824	0.5523	0.8957	0.1498
		SB	0.2542	0.5037	0.8610	0.1248
		PB	0.2613	0.5069	0.8743	0.1289
		BC	0.2602	0.5043	0.9400	0.1284
		BT	0.2867	0.5536	0.8805	0.1555

x_0=true concentration value;
SB=standard bootstrap;
PB=percentile bootstrap;
BC=bias-corrected percentile bootstrap;
BT=bootstrap-t.

Concluding remarks

The results show that the performances of the bootstrap-t and of the Delta method are comparable in the context of quantitative real-time PCR assays. The findings are coherent with those obtained by Jones and Rocke (8) in a fictitious example of linear calibration.

One can argue that bootstrapping implies more complicated and time-consuming computation than the Delta method. However, as the accuracy of the delta confidence interval depends on the asymptotic normality of $\hat{x}_0$ and this assumption may be questionable in small samples like those currently used in PCR experiments, the bootstrap-t method appears to offer a reliable way of constructing a confidence interval that does not depend on this assumption.

Future investigations will evaluate the impact of possible heteroschedasticity on the construction of bootstrap confidence intervals.

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