Reference Genes for Quantitative Real-time Polymerase Chain Reaction (qPCR)) Analyses in Freshly Isolated Monocytes of Septic Patients

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Reference genes for Quantitative Real-Time Polymerase Chain Reaction (qPCR) analyses in freshly isolated monocytes of septic patients

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ABSTRACT

Background: Sepsis is a life-threatening organ dysfunction associated with unregulated host response to infection. About 20 million people develop sepsis annually, and up to 50% die. Monocytes and macrophages play a key role in the innate and adaptive immune responses but the fully role of these cells in patients with sepsis still remains to be investigated. One of the limitations for the studies of gene expression in monocytes/macrophages in sepsis is the choice of the reference genes. We determined herein the most stable internal gene (s) to investigate gene expressions in monocytes/macrophages of septic patients.

Methods: The expression stability of fifteen commonly used reference genes was analyzed by determining the comparative threshold cycle (Ct) values, using the BestKeeper, GeNorm, and NormFinder algorithms.

Results: BestKeeper analysis revealed that the syntaxin 5 (STX5A) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) genes are highly stable. GeNorm pointed out STX5A and phosphoglycerate kinase 1 (PGK1) as the most suitable combination, whereas through NormFinder glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 14-3-3 zeta/delta protein (YWHAZ) was the most stable combination. All program analyses discarded the use of heterogeneous nuclear ribonucleoprotein A/B (HNRNPAB). GeNorm and NormFinder indicated actin-beta (ACTB) as the minor stable gene.

Conclusions: The combined data indicated that STX5A, PGK1, GAPDH, and HPRT1 are highly suitable reference genes for qPCR analysis of septic patient monocytes. In choosing one reference gene, the results point out STX5A (first place by GeNorm and BestKeeper and third place by NormFinder). This study is the first report on reference genes in freshly obtained monocytes/macrophages from septic patients.

Keywords: sepsis, macrophage, RNA analysis, STX5A, PGK1, GAPDH, and HPRT1.

BACKGROUND

Monocytes and macrophages are the front-line innate defense cells against pathogens [1,2]. Monocytes are immature immune cells circulating in the bloodstream, and they are locally recruited and become mature macrophages when there is tissue injury [3]. Macrophages are plastic cells and can differentiate into pro-inflammatory (M1) or anti-inflammatory and tissue repair (M2) types according to the microenvironment[3,4]. Monocytes and macrophages play an essential role in immunomodulation that occurs during sepsis [5]. According to the most recent definition (Sepsis-3; 2016), sepsis is a life-threatening organ dysfunction associated with unregulated host response to infection. Septic shock is a shock subset that exhibits circulatory and metabolic dysfunctions with high mortality risk [6]. About 20 million people develop sepsis annually, and 30%-50% die. Sepsis is still a serious public health problem [7,8].

Monocytes and macrophages have a set of receptors known as pattern recognition receptors (PRR), which rapidly initiate the inflammatory response upon tissue damage or in the presence of pathogens. The toll-like receptors (TLRs) are a subfamily of PRRs and are the major cellular components required for pathogen-associated molecular patterns (PAMPs) recognition and inflammatory response initiation. Among TLRs, TLR4 mediates lipopolysaccharide (LPS) recognition in the cell wall of gram-negative bacteria. Lipoteichoic acid and glycan peptides found in gram-positive bacteria activate TLR2. The activation of the TLR2 triggers an inflammatory response in sepsis [9,10], and systemic activation of the immune response occurs due to the release of the PAMPs or DAMPs (damage-associated molecular patterns) from microorganisms or lesioned tissue, respectively [9].

A few studies reported monocyte/macrophage gene expression results in septic conditions using different experimental approaches, mostly *in vitro* studies using murine bone marrow-derived macrophages and J774A1 murine macrophage cell line [11-16]. The followings were used as reference genes in the mentioned studies: 18S (18S RNA ribosomal), ACTB (beta actin), B2M (beta 2-microglobulin), GAPDH (glyceraldehyde 3-phosphate dehydrogenase), GUSB (glucuronidase beta), HMBS (hydroxymethylbilane synthase), HNRNPAB (heterogeneous nuclear ribonucleoprotein

A/B), HPRT1 (hypoxanthine phosphoribosyltransferase 1), MAU2 (MAU2 chromatid cohesion factor homolog), PGK1 (phosphoglycerate kinase 1), PPIA (peptidyl-prolyl isomerase A), PPIB (peptidyl-prolyl cis-trans isomerase B), RPL13A (ribosomal protein L13A), STX5A (syntaxin 5), YWHAZ (14-3-3 protein zeta/delta). So far, there is no consensus regarding reference genes to normalize monocyte/macrophage qPCR results in sepsis regardless of the experimental approach.

Several parameters ensure reliability and reproducibility in the quantitative gene expression measurements. The mentioned parameters are variations in initial sample volume, RNA integrity, cDNA synthesis efficiency, and differences in overall transcriptional activity in tissues or cells analyzed [17,18]. The most widely used approach to the normalization of the interest gene expression is an inner control gene. The internal control gene would be the one that detection is constant and continued in all tissues. The internal control gene expression does not change in any experimental condition and treatment, allowing the normalization of the interest gene [17-21]. Usually, there are one or more genes constitutively expressed. Firstly, it is necessary to identify potential candidates and test their expression stability in the condition under investigation [17,20]. Every mRNA analysis requires stably expressed reference genes as control. Reproducibility and reliability of the qPCR results demand reference genes [20]. Herein, we determined the most stable internal genes in monocytes/macrophages freshly obtained from septic patients.

RESULTS

Figure 1 exhibits individual Ct values of candidate reference genes across the nine plasma monocyte samples from septic patients. Candidate reference genes presented different Ct's values with 18S showing the highest mRNA expression (CT of 10 ± 1.15; mean ± SD) and STX5A the lowest (CT of 32 ± 0.12; mean ± SD).

Expression profile of candidate reference genes using the BestKeeper software

BestKeeper software determines the stability of the candidate genes by performing a pair-wise comparative analysis based on crossing points, and the best stability values are indicated by the lowest standard deviation and coefficient of variance [22]. The BestKeeper software identified the STX5A as the most stable and 18S as the less stable gene among 15 evaluated (Table 3) considering their standard deviation and coefficient of variance values.

Expression profile of the 15 reported reference genes using the GeNorm software

We tested the Ct values of the 15 genes obtained by qPCR analysis on GeNorm software that classified them according to the average measures of expression stability (M value). The most stable gene has the lowest M-value ($M \leq 0.5$), whereas values close to the upper limit of 1.5 are the minor stable genes [20]. Seven candidate genes presented $M \leq 0.5$: STX5A, PGK1, GAPDH, GUSB, HPRT1, YWHAZ, and B2M. The program calculates the Vn/n+1 between two sequential normalization factors to determine the optimal number of reference genes for normalization. For the cut-off value of V=0.15, two genes are required for accurate normalization. The analysis indicated STX5A and PGK1 as the best reference genes based on the high stability and low combined variation (Figure 2).

Expression profile of the 15 reported reference genes using the NormFinder algorithm

The NormFinder algorithm allows estimating the gene expression variation intergroup and intragroup. High stability of gene expression is indicated by a low stability value associated with the systematic error of each reference gene [17]. The best stability value is almost equal to zero. The ACTB and HNRNPAB genes exhibited higher expression stability values (Table 4). The NormFinder analysis indicated the GAPDH as the most suitable single gene for qPCR normalization in sepsis and GAPDH and YWHAZ as the most stable combination.

Ranking of the 15 reported reference genes as indicated by the three software tools

According to the BestKeeper analysis, the expression of the STX5A, HPRT1, and PGK1 genes was more stable, with the lowest standard deviation values and coefficient of variance. The NormFinder analysis indicated that a combination of GAPDH and YWHAZ has high expression stability. The GeNorm classified STX5A and PGK1 as the most top stable genes with a low combinatorial variation. Expressions of ACTB, HNRNPAB, and 18S genes were considered the least stable ones, according to the highest M value (GeNorm), coefficient of variance (BestKeeper), and stability value (NormFinder), respectively (Table 5).

DISCUSSION

Only a few studies reported gene expression results in monocytes/macrophages in experimental sepsis approaches (Table 1). The authors of the mentioned studies performed in vitro experiment using mice bone marrow-derived monocytes/macrophages [19,23], J774A1 murine macrophage cell line [24], monocytes from healthy donors stimulated with LPS [25], and monocytes from patients with relapsing-remitting multiple sclerosis [26]. Fifteen different genes were used as reference genes to normalize qPCR results in monocytes/macrophages under the experimental sepsis conditions mentioned. We then determined the most appropriate reference genes for studies in monocyte/macrophage freshly obtained from septic patients.

In response to several stimuli as LPS, lipoteichoic acid, and glycan peptides, the expression of several genes modifies in monocytes [2]. The expression of reported reference genes in macrophages might also change in critical clinical conditions such as sepsis [22]. Thereby, to investigate changes in expression of the interest genes, it is first necessary to determine which reference gene expression is stable in monocytes/macrophages of septic patients.

A previous report using LPS stimuli in 6 hours cultured macrophages indicated the HNRNPAB as the most stable reference gene [19]. Two other studies reported ACTB as a reference gene in monocytes from septic patients [11,13]. We reported HNRNPAB and ACTB as two of the minor stable genes in

freshly obtained monocytes/macrophages from septic patients, according to GeNorm, NormFinder, and BestKeeper tools.

The other two reports used 18S as a reference gene to normalize interest gene expressions in monocytes [15,27]. The NormFinder and BestKeeper analysis indicated 18S is the most unstable of the reference genes in freshly obtained monocytes/macrophages under the conditions of this study.

The use of GAPDH to normalize gene expression was reported in mononuclear cells from septic patients [16] and in an experimental septic model using immortalized monocyte [28]. GAPDH was considered herein as the most stable gene by the NormFinder analysis. Cummings et al. [29] evaluated the expression of HPRT1 and GAPDH in the whole blood of septic patients and identified that HPRT1 is more stable and reliable for data normalization than GAPDH. Our results indicate that GAPDH is a reliable gene for normalizing monocyte gene expression data; perhaps this is a result of the different samples and experimental conditions used.

Another study with monocytes from critically ill patients and septic patients used the geometric mean of five target genes with the minor variation as reference genes[12]. As mentioned, most of these studies used *in vitro* LPS-stimulated monocytes, which differ from cells freshly obtained from septic patients. Coulibaly et al. [30] identified that AKIRIN1 is a potential reference gene for sepsis. However, the cells studied were granulocytes and natural killer (NK) cells. In certain conditions, such as LPS stimulation, expressions of ACTB or GAPDH are modulated and cannot be considered good normalizers of gene expression in these samples [24].

We identified herein STX5A, PGK1, GAPDH, and HPRT1 as the most stable reference genes analyzing gene expression in monocytes freshly obtained from septic patients. In choosing one reference gene, the results point out STX5A; first place by GeNorm and BestKeeper and third place by NormFinder of the genes investigated. This study is the first report on reference genes in freshly obtained monocytes/macrophages from septic patients.

METHODS

Literature search strategy

We used the National Center for Biotechnology Information (NCBI) public PubMed database (http/www.ncbi.nlm.nih.gov/pubmed) for article search. We defined combinations using SEPSIS, SEPTIC SHOCK, MONOCYTE, MACROPHAGE, REFERENCE GENE, and GENE EXPRESSION.

Selection of samples

We selected samples from nine patients with a clinical diagnosis of sepsis admitted to the Intensive Care Unit (ICU) of the University Hospital of the University of Sao Paulo in the city of Sao Paulo, Brazil. Samples from septic patients were collected whenever allowed by the clinicians up to 4 days of hospitalization. The number of samples reached the recommendations for the GeNorm tool analysis [20].

Monocyte preparation

We collected 20 mL of blood in tubes containing EDTA from septic patients. Samples were centrifuged at 400 x g to obtain cells. We diluted the cells in phosphate buffer saline pH 7.4 and added Histopaque 1077 (Sigma Chemical Co, St Louis, MO, USA). We centrifuged the tubes at 400 x g and 4 °C for 30 minutes resulting in the isolation of mononuclear cells, which were cultured for one hour in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich) at 37 °C in a 5% CO2 incubator. Following incubation, the monocytes adhered to the culture plate were isolated in Trizol® (Invitrogen, Carlsbad, California, EUA) and stored at -80 °C for further analysis.

RNA isolation and cDNA synthesis

According to the manufacturer protocol, total RNA was extracted from monocytes using RNAqueous Microkit® (Invitrogen, Carlsbad, California, USA). The determination of the concentration and purity of the RNA samples was performed by absorbance analysis at 260/280 nm using a Thermo Scientific NanoDrop (Thermo Fisher Scientific, Waltham, Massachusetts, USA). cDNA was synthesized from the extracted RNA (1000 μg) using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). We used a similar procedure in previous studies [31].

Selection of candidates for reference genes

We first listed 15 reference genes previously used in studies with monocytes/macrophages from different sepsis experimental approaches, primarily in bone marrow-derived cells and the J7741 murine macrophage cell line (Table 1). We obtained specific primers for each gene using the National Center for Biotechnology Information (NCBI) public Gene Bank database (http/www.ncbi.nlm.nih.gov/genbank). We used the Primer-BLAST program (NCBI, Bethesda, Maryland, USA) to design the primers (Table 2). The hybridization temperature of the primers was standardized for the experiment (60°C).

qPCR

We performed the qPCR using QuantStudio 3 (ThermoFisher, Waltham, Massachusetts, EUA). The 25 μL reaction mix had 12.5 μL Power SYBR Green PCR Master Mix (ThermoFisher, Waltham, Massachusetts, EUA); 40 ng cDNA, and one μM forward and reverse primers (EXXTEND, Paulínia, São Paulo, Brazil); and 6.9 μL nuclease-free water, added to a 96-well plate. The cycling conditions followed the protocol of the SYBR Green manufacturer. We analyzed the melt curve to determine the specificity of the qPCR products and the absence of primer-dimer formation. We used a similar procedure in previous studies [31]. We followed the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines [32].

Bioinformatic tools used

The expression stabilities of the reference genes were determined using the BestKeeper (www.gene-quantification.de/bestkeeper.html, version 1), GeNorm (www.qbaseplus.com, version 3.2), and NormFinder (moma.dk/Normfinder-software, version 0.953) software. The BestKeeper software uses the coefficient of gene expression variance to analyze which reference gene is the most stable one [22]. The GeNorm algorithm determines the stability by calculating the arithmetic mean of all pair-wise variations of a particular reference gene relative to another[20]. The NormFinder algorithm classifies genes according to their expression stability in a given collection of samples and experimental design[17].

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DECLARATIONS

Ethics approval and consent to participate

All participants included in this study were over 18 years old, and all signed the informed consent form. The Ethics Committee of the University of São Paulo Hospital (HU-USP-Process number 1513 of 29th January 2016) approved the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Competing interests

The authors declared that no conflict of interest exists.

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Authors' contributions

Design of the study: RBG, TSS, LNM, FGS, MCCM, and RC. Collection of the samples: RBG and TSS. Laboratory measurements: RBG and TSS. qPCR analysis: RBG. Software analysis: RBG, TSS, and JFG. Interpretation of the findings and writing the manuscript: RBG, TSS, LNM, JFG, GMM, FGS, RBA, MCCM, TCPC, and RC. All authors approved the final manuscript version for publication and accepted accountabilities for all aspects of the work and for authorship.

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ABBREVIATIONS

18S: 18S RNA ribosomal

ACTB: Beta actin

B2M: Beta 2-microglobulin

BMDMS: Murine bone marrow-derived macrophages

cDNA: Complementary DNA

COPD: Patients with chronic obstructive pulmonary disease

CT: Threshold cycle

ER: Endoplasmic reticulum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GUSB: Glucuronidase beta

HMBS: Hydroxymethylbilane synthase

HMØ: Human monocytes stimulated with LPS

HNRNPAB: Heterogeneous nuclear ribonucleoprotein A/B

HPRT1: Hypoxanthine phosphoribosyltransferase 1

ICU: Intensive Care Unit

LPS: lipopolysaccharide

MAU2: MAU2 chromatid cohesion factor homolog

Messenger RNA: mRNA

NCBI: National Center for Biotechnology Information

PAMPS: Pathogen-associated molecular patterns

PGK1: Phosphoglycerate kinase 1

PPIA: Peptidylprolyl isomerase A

PPIB: Peptidyl-prolyl cis-trans isomerase B

PRR: Pattern Recognition Receptors

qPCR: Quantitative of polymerase chain reaction

RNA: ribonucleic acid

RPL13A: Ribosomal protein L13a

RRMS: Remitting multiple sclerosis

STX5A: Syntaxin 5

THP-1: Human THP-1 monocytic leukemia cell line

TLR2: Toll-like receptor 2

TLR4: Toll –like receptor 4

TLRs: toll-like receptors (TLRs)

YWHAZ: 14-3-3 protein zeta/delta

FIGURE AND LEGENDS

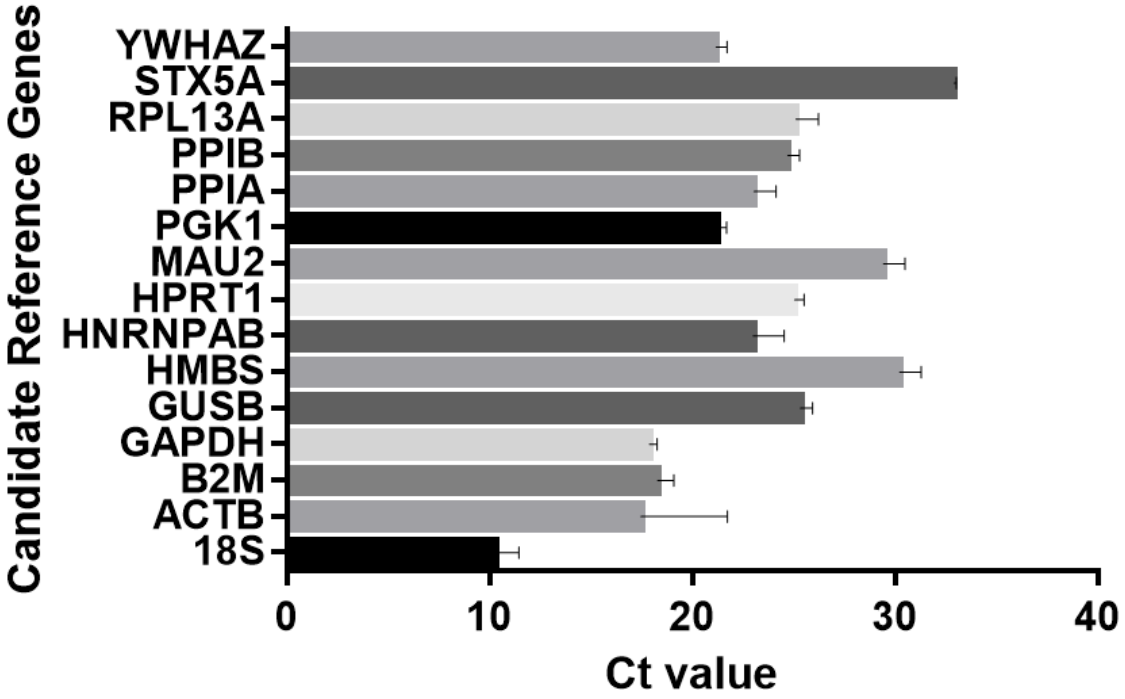


Figure 1. Cycle quantification (Ct) values of reference genes in plasma monocytes of septic patients harvested during hospitalization and immediately after hospital ICU discharge. The bars represent the average with standard deviation.

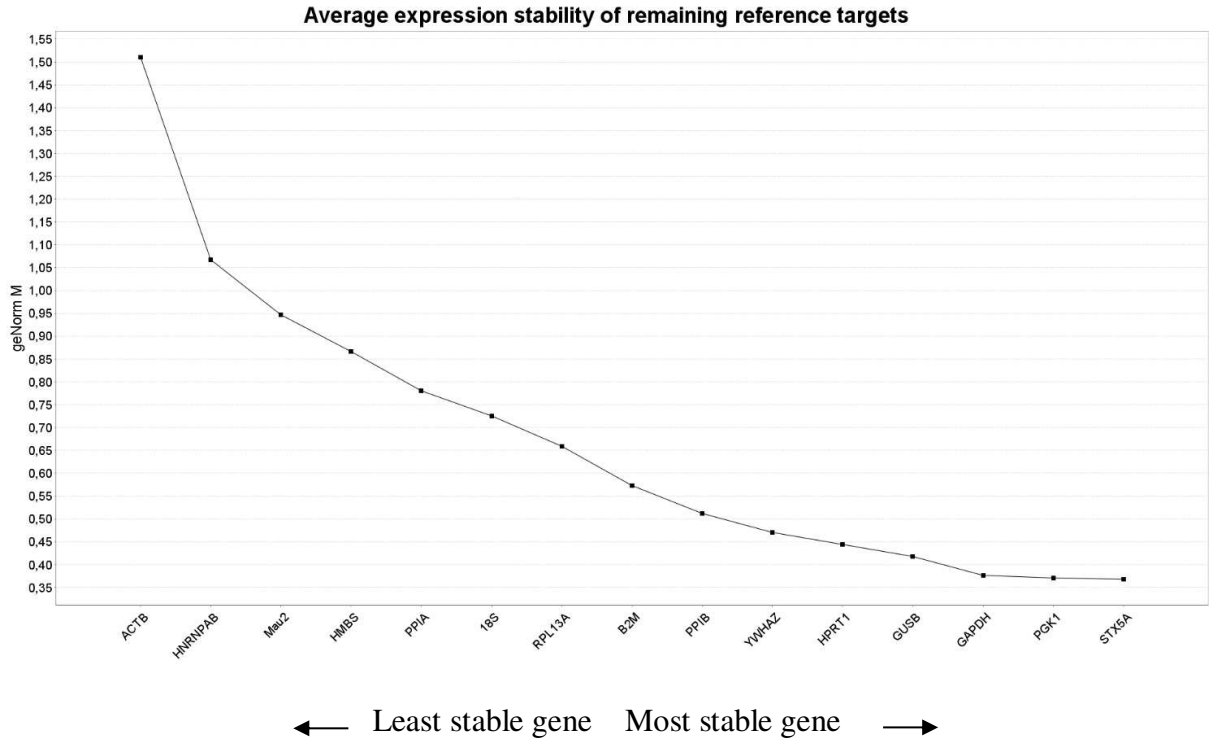


Figure 2. Average values (M) of expression stability of the 15 reported reference genes analyzed by using the GeNorm algorithm. This graph displays the output of GeNorm plotting the M value against the reference genes considering all samples from each group. Genes with M≤ 0.5 are considered as optimal reference [20].

TABLES

GENE	NAME	LOCATION	FUNCTION*	MODEL	REFERENCE
18S	18S RNA ribosomal	Ribosome	Component of eukaryotic cytoplasmic ribosomes	Mice blood monocyte	27
ACTB	Beta actin	Cytoeskeleton, Nucleus, Cytosol	Cytoskeletal structural actin	BMDMs, J774A1, COPD, THP-1	19,24,33,34
B2M	Beta 2-microglobulin	Plasma membrane, ER, Cytosol	Component of the MHC class 1 molecules	BMDMs, J774A1, COPD, THP-1, HMØ	19,23-25,33,34
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Cytosol	Glycolytic enzyme	BMDMs, J774A1, COPD, THP-1, HMØ, RRMS	19,23-26,33,34
GUSB	Glucuronidase beta	Extracellular, Lysosome	Hydrolase that degrades glycosaminoglycans	BMDMs, J774A1, THP-1	19,24,34
HMBS	Hydroxymethylbilane synthase	Cytosol	Production of heme	BMDMs	19
HNRNPAB	Heterogeneous nuclear ribonucleoprotein A/B	Nucleus	mRNA processing	BMDMs	19
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Cytosol	Generation of purines	BMDMs, J774A1, COPD, RRMS	19,23,24,26,33
MAU2	MAU2 chromatid cohesion factor homolog	Nucleus, Cytosol	Cell cycle	BMDMs	19
PGK1	Phosphoglycerate kinase 1	Cytosol	Glycolytic enzyme	J774A1	24
PPIA	Peptidylprolyl isomerase A	Extracellular, Cytosol	Accelerate the folding of proteins	BMDMs, J774A1, HMØ	19,23-25
PPIB	Peptidyl-prolyl cis-trans isomerase B	Nucleus, ER	Folding of collagen type I proteins	THP-1, HMØ	25,34
RPL13A	Ribosomal protein L13a	Cytosol	Component of the 60S ribosomal subunit	BMDMs, J774A1	19,23,24
STX5A	Syntaxin 5	Golgi, ER	Autophagy	BMDMs	19
YWHAZ	14-3-3 protein zeta/delta	Nucleus, Cytosol	Signaling pathways	RRMS	26

Table 1. List of 15 reported reference genes in different sepsis experimental approaches. *Source Gene Function: GeneCards [30]. ER: endoplasmic reticulum. LPS: lipopolysaccharide. Murine bone marrow-derived macrophages (BMDMs). J774A1 murine macrophage cell line. Patients with chronic obstructive pulmonary disease (COPD). Human THP-1 monocytic leukemia cell line (THP-1). Human monocytes stimulated with LPS (HMØ). Samples of patients with relapsing-remitting multiple sclerosis (RRMS).

GENE	FORWARD SEQUENCE	REVERSE SEQUENCE
B2M	GATGAGTATGCCTGCCGTGT	CTGCTTACATGTCTCGATCCCA
YWHAZ	CGCTATGAAGGCGGTGACAGA	TGACCCTCCAGGAAGATCGC
PPIB	AACGCAACATGAAGGTGCTC	GTAGGTCAAAATACACCTTGACGG
PPIA	GTTCTTCGACATTGCCGTCG	TGCCATCCAACCACTCAGTC
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
GAPDH	TTCAACAGCGACACCCACT	TTCCTCTTGTGCTCTTGCT
HMBS	ACCTCCTTCCCTCATACAGCA	ACGAGCAGTGATGCCTACCA
RPL13A	CCTTCCTCCATTGTTGCCCT	TGCACAATTCTCCGAGTGCT
STX5A	GAACACGGATCAGGGTGTCTA	ACGTTCTCGTCGATCCTCTG
GUSB	ATGCCATCGTGTGGGTGAAT	TGGCGATAGTGATTCGGAGC
HNRNPAB	GGCCGTGTCATTGACCCTAA	TCAATCTCCCCAAACTCGCC
HPRT1	CCTGGCGTCGTGATTAGTGA	CGAGCAAGACGTTCAGTCCT
ACTB	CTTCGCGGGCGACGAT	CCACATAGGAATCCTTCTGACC
PGK1	CCACTGTGGCTTCTGGCATA	ATGAGAGCTTTGGTTCCCCG
MAU2	AGGACAAGGTCGCAGAAAGG	ACGCAACCAACCAATTGAGC

Table 2. Primers sequences of the 15 reported reference genes.

	N	*NT*	*GM*	*AM*	*MIN*	*MAX*	*SD*	*SED*	*CV*
STX5A	*9*	*2*	*32.91*	*32.91*	*32.80*	*33.14*	*0.13*	*0.04*	*0.39*
HPRT1	*9*	*2*	*25.05*	*25.05*	*24.54*	*25.73*	*0.46*	*0.15*	*1.85*
PGK1	*9*	*2*	*21.25*	*21.26*	*20.78*	*22.30*	*0.44*	*0.15*	*2.05*
GAPDH	*9*	*2*	*17.89*	*17.89*	*17.25*	*18.32*	*0.39*	*0.13*	*2.19*
GUSB	*9*	*2*	*25.34*	*25.34*	*24.15*	*26.08*	*0.59*	*0.20*	*2.33*
PPIB	*9*	*2*	*24.73*	*24.73*	*23.82*	*25.53*	*0.58*	*0.19*	*2.34*
YWHAZ	*9*	*2*	*21.18*	*21.19*	*20.26*	*21.94*	*0.53*	*0.18*	*2.50*
HMBS	*9*	*2*	*30.23*	*30.24*	*28.47*	*32.32*	*1.07*	*0.36*	*3.53*
B2M	*9*	*2*	*17.98*	*17.99*	*17.26*	*18.94*	*0.66*	*0.22*	*3.66*
MAU2	*9*	*2*	*29.41*	*29.42*	*27.82*	*31.68*	*1.08*	*0.36*	*3.67*
RPL13A	*9*	*2*	*25.10*	*25.12*	*23.01*	*26.80*	*1.12*	*0.37*	*4.48*
PPIA	*9*	*2*	*23.03*	*23.06*	*21.98*	*25.53*	*1.08*	*0.36*	*4.68*
ACTB	*9*	*2*	*19.42*	*19.45*	*18.05*	*22.58*	*1.28*	*0.43*	*6.56*
HNRNPAB	*9*	*2*	*22.96*	*23.00*	*21.60*	*26.86*	*1.55*	*0.52*	*6.73*
18S	*9*	*2*	*10.25*	*10.30*	*8.94*	*12.19*	*1.15*	*0.38*	*11.20*

Table 3. Bestkeeper software evaluation of the 15 reported reference genes . N = number of samples, NT = number of replicates of each sample, GM = geometric mean, AM = arithmetic mean, Min = minimal value, Max = maximal value, SD = standard deviation, CV = coefficient of variance (%). STX5A is the most stable gene and 18S is the least.

Gene	Stability Value
GAPDH	0.027
YWHAZ	0.077
STX5A	0.079
HPRT1	0.098
PGK1	0.130
GUSB	0.149
B2M	0.169
PPIB	0.171
PPIA	0.273
HMBS	0.281
MAU2	0.334
18S	0.351
RPL13A	0.355
ACTB	0.442
HNRNPAB	0.445

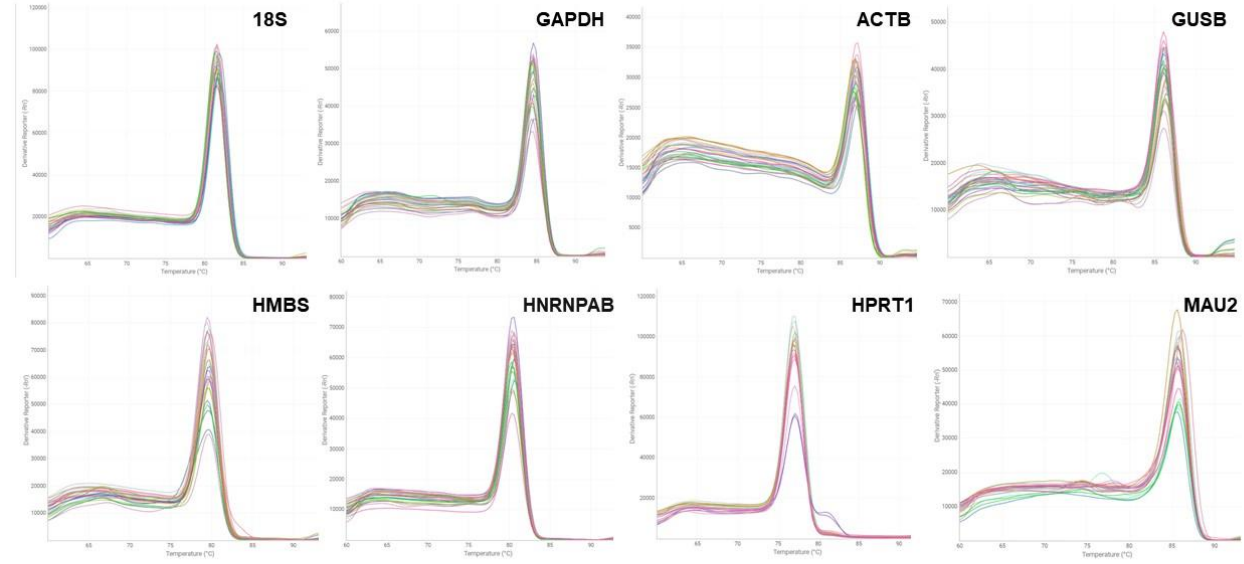
Table 4. Crescent stability of the 15 reported reference genes determined by the NormFinder algorithm.

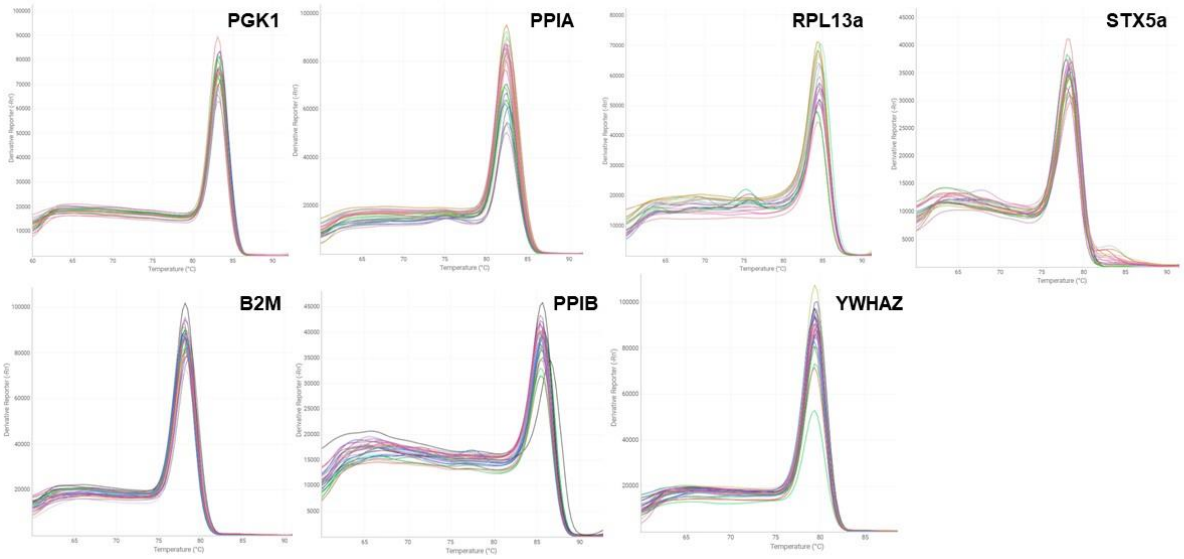
RANKING	*GeNorm*	*NormFinder*	*BestKeeper*
Most Stable	STX5A	GAPDH	STX5A
	PGK1	YWHAZ	HPRT1
	GAPDH	STX5A	PGK1
	GUSB	HPRT1	GAPDH
	HPRT1	PGK1	GUSB
	YWHAZ	GUSB	PPIB
	B2M	B2M	YWHAZ
	PPIB	PPIB	HMBS
	RPL13A	PPIA	B2M
	18S	HMBS	MAU2
	PPIA	MAU2	RPL13A
	HMBS	18S	PPIA
	MAU2	RPL13A	ACTB
	HNRNPAB	ACTB	HNRNPAB
Least Stable	ACTB	HNRNPAB	18S

Table 5. Ranking of the 15 reported reference genes in monocytes from septic patients.

SUPPLEMENTARY MATERIAL

We used the melting curve to determine the specificity of the primers. The presence of a single amplification peak indicates the specificity of the primers used.





Figures

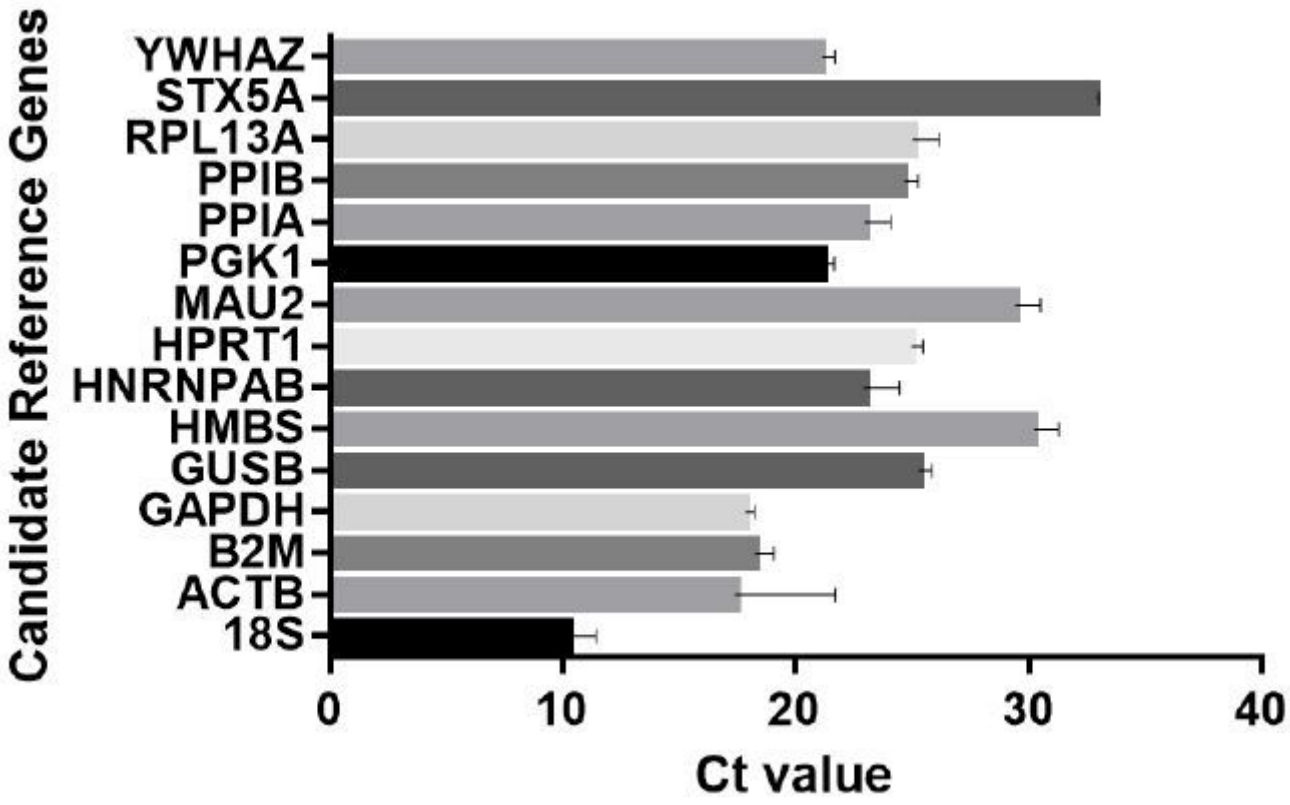


Figure 1

Cycle quantification (Ct) values of reference genes in plasma monocytes of septic patients harvested during hospitalization and immediately after hospital ICU discharge. The bars represent the average with standard deviation.

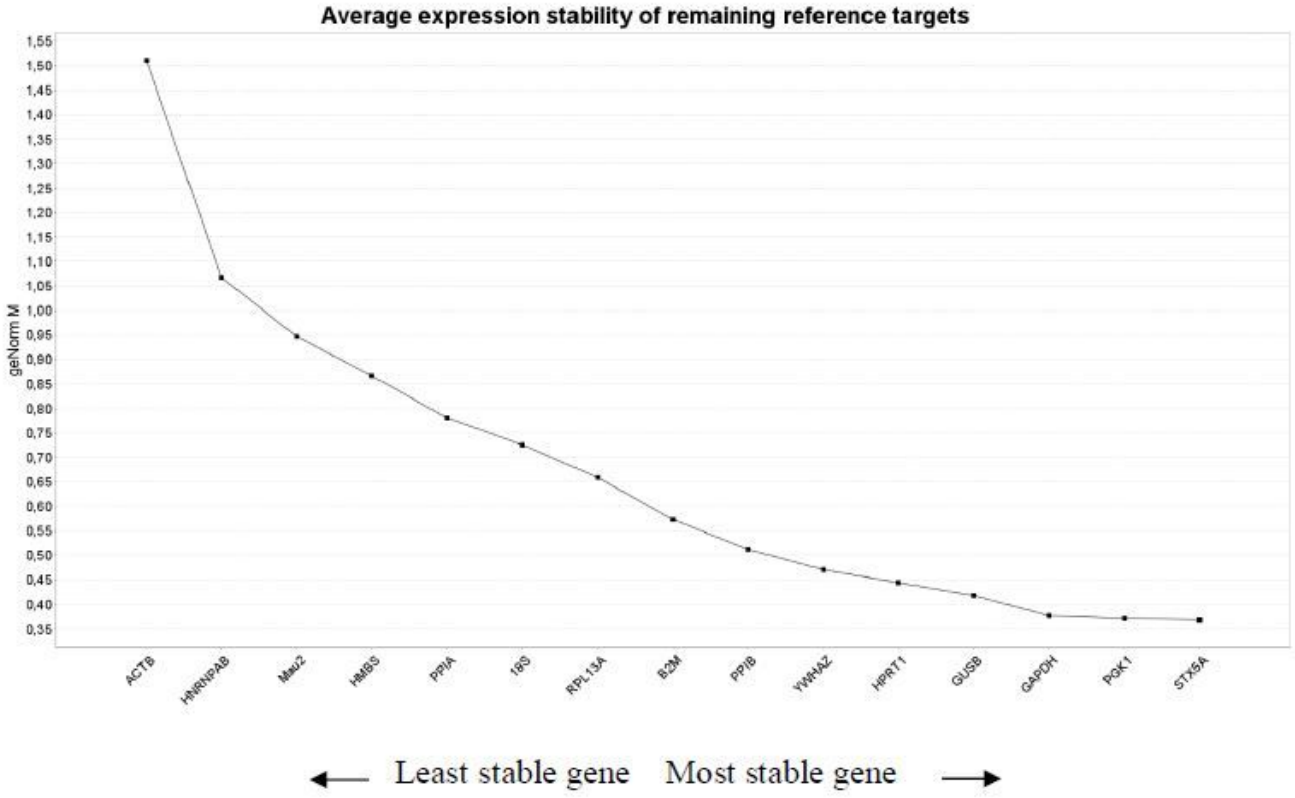


Figure 2

Average values (M) of expression stability of the 15 reported reference genes analyzed by using the GeNorm algorithm. This graph displays the output of GeNorm plotting the M value against the reference genes considering all samples from each group. Genes with M≤ 0.5 are considered as optimal reference 20.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supp.docx