Biomedical Science Letters 2023, 29(4): 220~230
https://doi.org/10.15616/BSL.2023.29.4.220
eISSN : 2288-7415

Original Article

Validation of Synovial Fluid Clinical Samples for Molecular Detection of Pathogens Causing Prosthetic Joint Infection Using *GAPDH* Housekeeping Gene as Internal Control

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Identification of the pathogens causing infection is important in terms of patient's health management and infection control. Synovial fluids could be used as clinical samples to detect causative pathogens of prosthetic joint infections (PJIs) using molecular diagnostic assays, therefore, normalization and validation of clinical samples are necessary. Microbial culture is considered the gold standard for all infections, including PJIs. Recently, molecular diagnostic methods have been developed to overcome the limitation of microbial culture. Therefore, guideline for validating clinical samples to provide reliable results of molecular diagnostic assays for infectious diseases is required in clinical field. The present study aimed to develop an accurate validating method of synovial fluid clinical samples using *GAPDH* gene as an internal control to perform the quantitative PCR TaqMan probe assay to detect pathogens causing PJIs.

Key Words: Prosthetic joint infections (PJIs), Synovial fluid (SF), Internal control, Molecular diagnosis

INTRODUCTION

Prosthetic joint infections (PJIs) are infections that usually affect joint prostheses and adjacent tissues. The microbiological basis for PJIs is clinically defined, with only a small proportion of postoperative infections undergoing microbiological sampling can identify the causative pathogen (Lamagni, 2014).

The incidence of PJIs after knee arthroplasty is estimated

Received: October 25, 2023 / Revised: November 15, 2023 / Accepted: November 15, 2023
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to be approximately 1~2% (Kapadia et al., 2016). PJI is a serious complication after implant surgery and reduces quality of life by causing functional problems in the joints after infection occurs (Lee et al., 2022; Vale et al., 2023; Karczewski et al., 2019). As knee osteoarthritis (OA) can be treated *via* orthopedic surgery, PJIs can be surgically treated using unicompartmental knee arthroplasty (UKA) or high tibial osteotomy (HTO). UKA offers superior survivability, while HTO can delay total knee arthroplasty (TKA) by improving joint alignment and preservation (Takeuchi et al., 2010; Fu et al., 2013). The criteria for determining PJIs are important, and the 2013 Musculoskeletal Infection Society (MSIS), 2018 International Consensus Meeting (ICM), and modified MSIS criteria are defined as the criteria for determining PJIs (Parvizi et al., 2018; Patel et al., 2023; Kuiper et al., 2022). Currently, the modified MSIS criteria, which include two major and several minor criteria, are most commonly used to determine PJIs. The modified MSIS includes positive cultures, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) of serum, white blood cell (WBC) count, leukocyte esterase (LE), polymorphonuclear neutrophil (PMN) from synovial fluid (SF), and histological analysis (Kuiper et al., 2022; Zmistowski et al., 2014).

Particularly, microbial culture tests are considered the gold standard for diagnosing all types of infections, including PJIs (Wouthuyzen-Bakker, 2023; Tarabichi et al., 2023). Novel molecular diagnostic technologies for the identification and detection of pathogens in PJIs have recently developed as a response to the limitations of microbial cultures. These technologies include specific real-time, broad-range, and molecular-level 16S rRNA-polymerase chain reaction (PCR), and next-generation sequencing (NGS) (Gatti et al., 2022). These approaches to overcome the limitations of cultures reduce the error rate and turnaround time of diagnostic methodologies (Gatti et al., 2022).

Several studies are being conducted on the normalization and validation of synovial fluid (SF) in molecular diagnostic methods using appropriate housekeeping genes (Nazet et al., 2019; Nakasa et al., 2008; Vandesompele et al., 2002). The housekeeping genes that are commonly used include β-actin (Kim et al., 2012; Blaschke et al., 2000), β-2 microglobulin (*B2M*) (Zhang et al., 2002), ribosomal protein L32 (*RPL32*) (Zhang et al., 2005), ribosomal protein, large, P0 (*RPLP0*) (Nazet et al., 2019), eukaryotic translation elongation factor 1 alpha 1 (*EEF1A1*) (Nazet et al., 2019), TATA-Box Binding Protein (*TBP*) (Nazet et al., 2019; Zhang et al., 2005), peptidylprolyl isomerase B (*PPIB*) (Nazet et al., 2019), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Nazet et al., 2019; Zhang et al., 2002; Zhang et al., 2005), and 18S rRNA (Nazet et al., 2019). Among these, GAPDH is the most widely used (Nazet et al., 2019; Nakasa et al., 2008; Kanbe et al., 2002; Xiong et al., 2018; Kasama et al., 2000; Fang et al., 2018) and a known glycolytic enzyme that catalyzes the conversion of glyceraldehyde 3-phosphate to 3-phospho glyceroyl phosphate and induces gluconeogenesis (Nazet et al., 2019; Ghasemi et al., 2017). *GAPDH* has been used as the internal control for normalizing differences for a target sample, particularly, to establish the relative expression of this target gene (Kanbe et al., 2002; Xiong et al., 2018). In another study using *GAPDH* primers as an internal control, quantitative PCR (qPCR) was performed, and non-amplified samples were excluded from case analysis (Fang et al., 2018).

Guideline for validating clinical samples to provide reliable results of molecular diagnostic assays is required in clinical field. Therefore, in the present study, the *GAPDH* gene design process involved designing new forward and reverse primers based on previously published research sites (Fang et al., 2018). This study aimed to develop an accurate validating method of synovial fluid clinical samples using *GAPDH* gene as an internal control to perform the qPCR TaqMan probe assay to detect pathogens causing PJIs.

MATERIALS AND METHODS

Collection of clinical specimens

A total of 113 SF clinical samples were collected from patients with symptomatic OA and who underwent TKA, HTO, or UKA at the Mokdong Himchan Hospital (Seoul, Republic of Korea) and Incheon Himchan Hospital (Incheon, Republic of Korea) from January 2022 to October 2023. This study was approved by the Institutional Review Board (IRB) of the Catholic University of Pusan (IRB Approval No.: CUPIRB-2022-01-006).

Table 1. Accession numbers and gene synthesis sequence of the *GAPDH* gene

Target gene	Organism	Gene accession No.	Gene synthesis sequence (Amplicon size; 230 bp)
GAPDH; glyceraldehyde-3-phosphate dehydrogenase (This study)	*Homo sapiens* (human)	J04038.1 NG_007073.2 AC006064.10 AY340484.1	GCGCTGCCAAGGCTGTGGGCAAGGTCATCCCTGAGC TGAACGGGAAGCTCACTGGCATGGCCTTCCGTGTCC CCACTGCCAACGTGTCAGTGGTGGACCTGACCTGCC GTCTAGAAAAACCTGCCAAATATGATGACATCAAGA AGGTGGTGAAGCAGGCGTCGGAGGGCCCCCTCAAG GGCATCCTGGGCTACACTGAGCACCAGGTGGTCTCC TCTGACTTCAACAGC

gDNA extraction from clinical samples

Genomic DNA (gDNA) was extracted from SF using the Puregene Blood Kit (Hilden, Germany). gDNA extraction was performed according to the manufacturer's protocol. A total of 1 mL SF was mixed with 5 mL cell lysis solution and 2 mL protein precipitation solution. The mixture was centrifuged at 2,000 × g for 10 min to precipitate protein pellets. The supernatants were mixed with 6 mL isopropanol and centrifuged at 2,000 × g for 10 min to discard the supernatant and extract a DNA pellet. After washing the DNA pellet with 6 mL of 70% ethanol, 200 μL DNA hydration solution was added to dissolve the DNA component. The samples were overnighted with gentle shaking at room temperature and subsequently transferred to an E-tube. The purity and concentration of all gDNA samples were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Middlesex County, MA, USA) and stored at -20℃ before further analysis.

Identification of microbial species isolated from SF samples

Microorganisms from the SF samples were identified using the VITEK 2 system (Biomerieux, La Balme les Grottes, France) according to the manufacturer's instructions.

Design of primer pairs and TaqMan probe

To develop a new internal control detection method for validating suitable SF samples, new primers and probes for *GAPDH* gene were designed. To detect *GAPDH* during this development process, the *GAPDH* sequences for accession no. J04038.1, NG_007073.2, AC006064.10, and AY-340484.1 of *Homo sapiens* were collected from GenBank (www.ncbi.nlm.nih.gov). Using the *GAPDH* sequence as internal control, a pair of primers and probes was designed for regions with no mismatch through multiple sequence alignment using Clustal Omega Tools (EMBL-EBI) (Table 1). After careful analysis of the *GAPDH* sequences, the primers were designed as forward and reverse primers. For the *GAPDH* gene target, the primer-probe sequences were evaluated for secondary structures and dimer-forming potential using the Oligo Analyzer software (Commercial Park Coralville, IA, USA). Then, the primer-probe sequence sets exhibiting the least secondary structures and dimer-forming potential were selected for use in qPCR and synthesized by the Macrogen Institute (Seoul, Republic of South Korea). The primers amplified approximately 290 bp of the *GAPDH*-specific fragment of GAPDH. Inside this region, the *GAPDH*-specific fluorescent probe was designed as the TaqMan probe.

Limit of detection (LOD) analysis

The synthesized plasmid *GAPDH* gene of the *Homo sapiens* species was used as an analytical sample to confirm the LOD of the primers and probe designed in this study. The plasmid gene was synthesized at Macrogen Institute (Seoul, Republic of Korea) by setting a specific target region of the GAPDH sequence. The absorbance for the synthesized gene was measured using a NanoDrop™ 2000 spectrophotometer (Middlesex County, MA, USA) and quantified using DNA Copy Number and Dilution Calculator, a web-based program that calculates the gene copy number based on the measured absorbance.

Table 2. Clinical data on patients and PCR and microbiology culture test results

	Gender		Age (Mean ± SD, years)	Surgical type			
	Male	Female		TKA	HTO	UKA	Non-surgery group
PJI (n=4, 3.5%)	3 (6.1%)	1 (1.6%)	55.5±20.65	3 (5.9%)	0 (0%)	0 (0%)	1 (1.8%)
Non-PJI (n=109, 96.5%)	46 (93.9%)	63 (98.4)	68.5±12.77	48 (94.1%)	1 (100%)	6 (100%)	54 (98.2%)
Total patients (n=113, 100%)	49 (100%)	64 (100%)	68.0±13.35	51 (100%)	1 (100%)	6 (100%)	55 (100%)

PJI, Prosthetic joint infection; SD, Standard deviation; TKA, Total knee arthroplasty; HTO, High tibial osteotomy; UKA, Unicompartmental knee arthroplasty

Primers and probe specificity tests analyzed by using qPCR TaqMan probe assay

To develop an internal control of *GAPDH* gene for validation of synovial fluid (SF) samples, specificity tests for target and non-target substances were conducted in this study. Optimized qPCR was performed on target and non-target substances to confirm that no substances other than the internal control GAPDH were detected in clinical SF samples. The target substance is the synthesized plasmid *GAPDH* gene and non-target substances are *Staphylococcus epidermidis* ATCC (American Type Culture Collection) 35989, *S. aureus* ATCC 29213, *Enterococcus faecalis* KCTC (Korean Collection for Type Cultures) 3511, *Streptococcus dysagalactiae* subsp. *equismilis* KCTC 3098, *S. pyogenes* ATCC 19615, *Escherichia coli* O157: H7 ATCC 35150, *Acinetobacter baumannii* KCTC 23254, *Pseudomonas aeruginosa* KCTC 22063, *Enterobacter cloacae* ATCC 2361, *Candida parapsilosis* KCTC 7653, *C. tropicalis* KCTC 7212, *C. glabrata* KCTC 7219, *C. albicans* ATCC 10231, and *Aspergillus fumigatus* KCTC 6145.

Optimization of annealing temperature for qPCR TaqMan probe assay

To confirm the detection of the *GAPDH* gene as internal control in SF, qPCR TaqMan probe assay was performed using the designed primers and probe in triplicate. Optimal qPCR compositions containing the designed primers and probes processed with template DNA samples were subjected to thermal cycling and fluorescence detection using the CFX-96 real-time PCR system (Hercules, CA, USA). qPCR amplification was performed in a total volume of 20.0 μL containing 2 × Thunderbird probe quantitative PCR mixture (Toyobo, Osaka, Japan), a forward primer, a reverse primer, the template DNA. Distilled water was added to make a final volume of 20.0 μL. Thermal cycling was performed at 95℃ for 10 min, followed by 40 cycles at 95℃ for 15 s, 58℃ for 30 s, and 72℃ for 30 s.

Statistical analysis

To determine differential expression levels of DNA, statistical analysis was performed using GraphPad Prism version 8 (La Jolla, CA, USA). The receiver operating characteristic (ROC) curve and area under the ROC curve (AUC) were used to evaluate the ability of SF DNA against the GAPDH gene as an internal control. The *P*-value <0.05 was considered to indicate a statistically significant difference.

RESULTS

Clinical samples collected from the hospitals

This study analyzed a total of 113 clinical SF samples collected from January 2022 to October 2023 from patients (49 male and 64 female) with a mean age of 68.0±13.35 years (range, 20~88 years). After aseptically preparing the joint area, the SF was aseptically aspirated from the joint cavity using a sterile needle. For this study, 1 mL SF was required and stored at -80℃ until further analysis (Table 2).

Amplification of *GAPDH* gene as internal control

To detect the *GAPDH* gene as an internal control in clinical SF samples, qPCR TaqMan probe assay was performed using the primers and probes designed in this study. The amplification signal was measured *via* qPCR using the CFX-96 real-time PCR system (Bio-Rad, Hercules, CA, USA). The cut-off value for the *GAPDH* gene was based

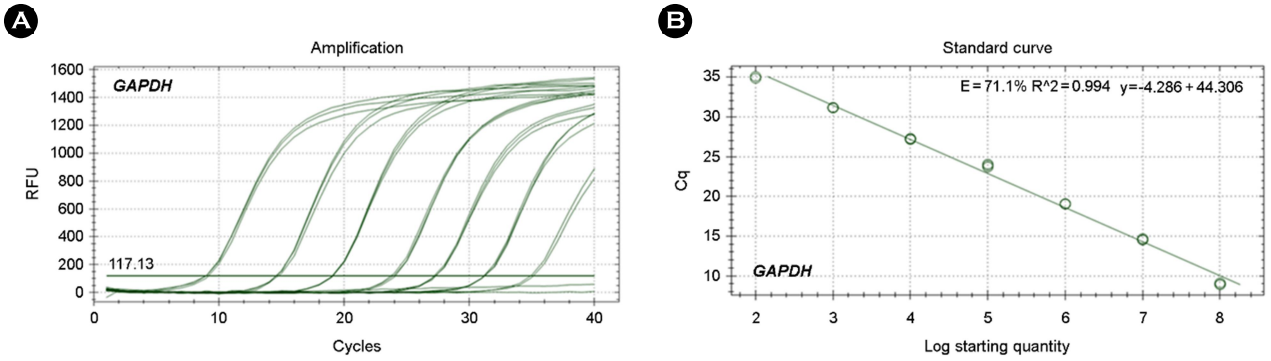

Fig. 1. Quantitative PCR TaqMan probe assay for *GAPDH* gene with concentration from 1×10^8 copies/μL to 1×10^1 copies/μL by 10-fold serial dilution. (A) Amplification curve (X-axis: Cycles, Y-axis: RFU, Relative Fluorescence Unit) and (B) Standard curve (X-axis: Log Starting Quantity, Y-axis: Cq, quantification cycle) of the target *GAPDH* gene plasmids.

Table 3. Mean Ct values for qPCR carried out on serial dilutions of plasmid DNA of the *GAPDH* gene. SD is standard deviation

No.	*GAPDH* plasmid				
	Plasmid DNA content	Mean Ct value	SD	Log value	Copies per reaction (copies/μL)
1	1.782 ng	8.99	0.10	8.24	1.74×10^8
2	178.2 pg	14.59	0.08	6.93	8.58×10^6
3	17.82 pg	19.06	0.02	5.89	7.78×10^5
4	1.782 pg	23.88	0.15	4.77	5.83×10^4
5	178.2 fg	27.23	0.06	3.98	9.66×10^3
6	17.82 fg	31.16	0.03	3.07	1.17×10^3
7	1.782 fg	34.97	0.11	2.18	1.51×10^2
8	178.2 ag	40.00	0.00	1.00	1.01×10^1

on the Ct value of 30, and clinical synovial samples with Ct values below 30 were classified as *GAPDH*-positive, whereas samples with Ct values above 30 were classified as *GAPDH*-negative.

qPCR TaqMan probe assay results for the *GAPDH* gene

To confirm the sensitivity of primers and probe designed in this study, the *GAPDH* plasmid was recombined via gene synthesis (Macrogen, Seoul, Republic of Korea) (Table 1). The recombinant plasmid was diluted 10 times in eight steps from 1×10^8 copies/μL to 1×10^1 copies/μL through serial dilution, and qPCR was performed in triplicate for each step. A standard curve was created based on the Ct value for amplification for each sample in eight steps, and each sample was quantified. The qPCR results showed that the R^2 value for the *GAPDH* plasmid was 0.994, indicating good linearity; however, the amplification efficiency was somewhat low at 71%. In addition, the detection limit for the target was confirmed to be 1×10^2 copies/μL. However, when the Ct value was verified in the clinical samples, Ct values over 30 were analyzed as inadequate results. Therefore, in this study, the *GAPDH*-negative cut-off value was set to ≥30 (Fig. 1, Table 3).

Cross-reactivity test of the quantitative assessment of target DNA

When detecting target strains by qPCR, no cross- with bacteria and fungi, which are infection-causing strains. To establish *GAPDH* as an internal control in clinical SF samples, the primers and probe designed in this study were used to verify its effectiveness and specificity. To determine the effectiveness and specificity of *GAPDH*, we used the

Table 4. Result of qPCR for *GAPDH* plasmid gene and reference strains in this study

Gene and reference strains			Control type	Real-Time PCR using design of primers and probe in This Study	
				Mean Ct value	Standard deviation
GAPDH gene	**The synthesized *GAPDH* gene (This study)†**		**Internal control**	**3.94**	**0.45**
Bacterial strains	ATCC 35989	*Staphylococcus epidermidis*	Negative control	N/A*	–
	ATCC 29213	*Staphylococcus aureus*	Negative control	N/A*	–
	KCTC 3511	*Enterococcus faecalis*	Negative control	N/A*	–
	KCTC 3098	*Streptococcus dysagalactiae* subsp. *equismilis*	Negative control	N/A*	–
	ATCC 19615	*Streptococcus pyogenes*	Negative control	N/A*	–
	ATCC 35150	*Escherichia coli* O157: H7	Negative control	N/A*	–
	KCTC 23254	*Acinetobacter baumannii*	Negative control	N/A*	–
	KCTC 22063	*Pseudomonas aeruginosa*	Negative control	N/A*	–
	ATCC 2361	*Enterobacter cloacae*	Negative control	N/A*	–
Fungal strains	KCTC 7653	*Candida parapsilosis*	Negative control	N/A*	–
	KCTC 7212	*Candida tropicalis*	Negative control	N/A*	–
	KCTC 7219	*Candida glabrata*	Negative control	N/A*	–
	ATCC 10231	*Candida albicans*	Negative control	N/A*	–
	KCTC 6145	*Aspergillus fumigatus*	Negative control	N/A*	–

*Not detection
†The following abbreviations are used: KCTC, Korean Collection for Type Cultures, Taejon, Korea; ATCC, American Type Culture Collection, Rockville, MD, USA

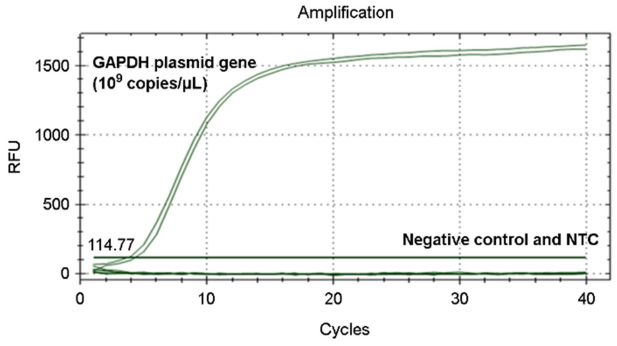

Fig. 2. Amplification plot of qPCR for the target synthesized *GAPDH* gene and negative sample including nine bacterial and five fungal reference strains. Amplification curves (X-axis: cycle, Y-axis: RFU, relative fluorescence unit) of the *GAPDH* plasmid gene as positive control and nine bacterial and five fungal reference strains as negative control and non-template control (NTC). Nine bacterial reference strains included *S. epidermidis*, *S. aureus*, *E. faecalis*, *S. dysagalactiae*, *S. pyogenes*, *E. coli*, *A. baumannii*, *P. aeruginosa* and *E. cloacae*. Five fungal reference strains included *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. albicans*, *A. fumigatus*.

GAPDH plasmid gene as positive control and 14 reference strains identified as the dominant species causing PJIs in clinical SF samples as negative control. Therefore, 14 reference strains were used as negative controls for qPCR. Among the nine bacterial reference strains included *S. epidermidis* ATCC 35989, *S. aureus* ATCC 29213, *E. faecalis* KCTC 3511, *S. dysagalactiae* subsp. *equismilis* KCTC 3098, *S. pyogenes* ATCC 19615, *E. coli* O157: H7 ATCC 35150, *A. baumannii* KCTC 23254, *P. aeruginosa* KCTC 22063, *E. cloacae* ATCC 2361, *C. parapsilosis* KCTC 7653, *C. tropicalis* KCTC 7212, *C. glabrata* KCTC 7219, *C. albicans* ATCC 10231, and *A. fumigatus* KCTC 6145. *GAPDH* gene was thus amplified by qPCR; however, the 14 dominant bacterial and fungal reference strains that caused PJI were not (Fig. 2 and Table 4).

Analysis of the VITEK 2 system

Identification of microorganisms in SF clinical samples collected from the Mokdong and Incheon Himchan Hospital was confirmed using the VITEK2 system (La Balme les Grottes, France) and the identification results were classified as culture-positive (CP) or culture-negative (CN). Out of

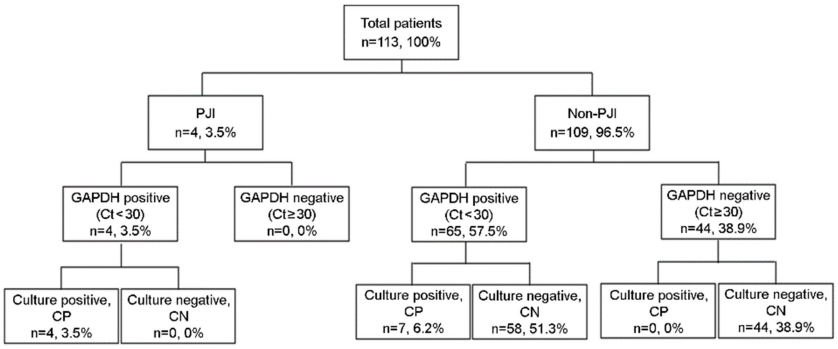

Fig. 3. Flowchart detailing enrolment and microorganism results and *GAPDH* results of study samples in PJI and non-PJI groups.

Table 5. Identification of microorganisms in clinical synovial fluid samples

	No.	Microorganism culture tests and identification using the VITEK2 system	Numbers of colonies in microbial culture
Mokdong Himchan Hospital	1	*Staphylococcus aureus* (Bacteria)	Many
	2	*Staphylococcus aureus* (Bacteria)	Many
	3	*Streptococcus dysagalactiae* (Bacteria)	Many
	4	*Escherichia coli* (Bacteria)	Moderate
	5	*Staphylococcus epidermidis* (Bacteria)	Few
	6	*Staphylococcus epidermidis* (Bacteria)	Few
	7	*Serratia marcescens* (Bacteria)	Moderate
	8	*Streptococcus dysagalactiae* (Bacteria)	Many
	9	*Streptococcus anginosus* (Bacteria)	Few
Incheon Himchan Hospital	10	*Klebsiella aerogenes* (Bacteria)	Few
	11	*Escherichia coli* (Bacteria)	Moderate

113 samples, 11 were culture-positive (CP) and 102 were culture-negative (CN). All 11 CP (100%, 11/11) samples were below the *GAPDH* cut-off value. However, 58 (56.9%, 58/102) CN samples were below the *GAPDH* cut-off value, whereas 44 (43.1%, 44/102) were above the *GAPDH* cut-off value. The bacterial species identified in the 11 CP samples were two cases of *S. aureus*, two of *S. epidermidis*, two of *S. dysagalactiae*, two of *E. coli*, one of *S. marcescens*, one of *S. anginosus*, and one of *Klebsiella aerogenes* (Fig. 3 and Table 5). Therefore, clinical SF samples from all 113 patients were categorized according to hospital, microbiology culture test results, PJI results, and *GAPDH* positivity. *GAPDH*-positive samples with a Ct value < 30 was confirmed in 100% of both the CP (n = 11) and PJI groups (n = 4), whereas 51.3% (n = 58) and 57.5% (n = 65) of the CN and non-PJI groups, respectively, were *GAPDH*-positive. However, no *GAPDH*-negative samples with Ct value ≥ 30 were found in the CP and PJI groups, whereas 44 cases in both the CN and non-PJI groups were *GAPDH*-negative, accounting for 39% of all patients (Tables 6 and 7).

Comparison of *GAPDH* level as an internal control between PJI, non-PJI, CP, and CN groups

To determine differences between PJI and non-PJI groups for *GAPDH*, we compared Ct values of the target DNA extracted from the SF samples. We found that the AUC and *P*-values of the Ct values for *GAPDH* gene in SF were AUC 0.8257 (**P* = 0.0322). In addition, the comparison between the CP and CN groups resulted in AUC 0.8975 (****P* < 0.0001) (Fig. 4).

Table 6. Clinical data on patients and qPCR and microbial culture test results

	Microbiology culture test		GAPDH	
	Positive	Negative	Positive	Negative
PJI (n=4)	4	0	4	0
Non-PJI (n=109)	7	102	65	44
Total patients (n=113)	11	102	69	44

PJI, Prosthetic joint infection; SD, Standard deviation; TKA, Total knee arthroplasty; HTO, High tibial osteotomy; UKA, Unicompartmental knee arthroplasty

Table 7. Summary of clinical sample for *GAPDH* gene

		GAPDH positive (Ct < 30) (n=69, 100%)	*GAPDH* negative (Ct ≥ 30) (n=44, 100%)	Total patients (n=113, 100%)
Hospital samples	Mokdong Himchan Hospital (number of patients)	31 (44.9%)	32 (72.7%)	63 (55.8%)
	Incheon Himchan Hospital (number of patients)	38 (55.1%)	12 (27.3%)	50 (44.2%)
Microbial culture tests results	Culture positive, CP (number of patients)	11 (9.7%)	0 (0.0%)	11 (9.7%)
	Culture negative, CN (number of patients)	58 (51.3%)	44 (39.0%)	102 (90.3%)
PJI results	PJI (number of patients)	4 (3.5%)	0 (0.0%)	4 (3.5%)
	non-PJI (number of patients)	65 (57.5%)	44 (39.0%)	109 (96.5%)

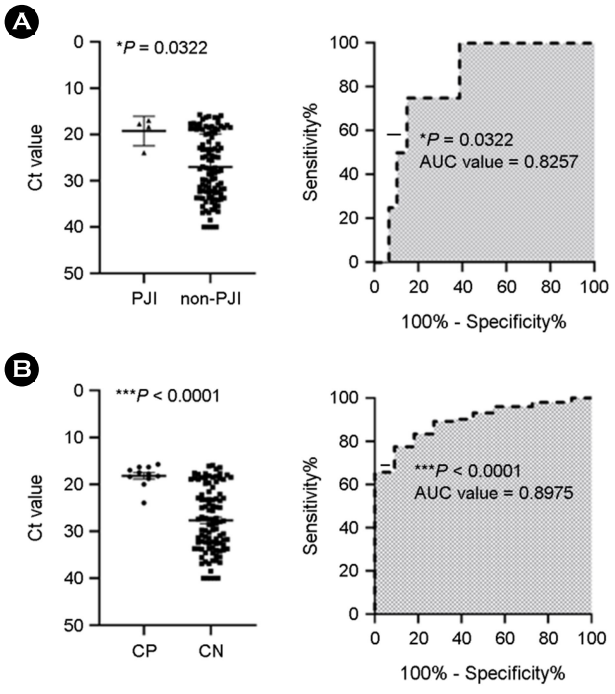

Fig. 4. The *GAPDH* gene for (A) PJI and non-PJI groups and (B) culture-positive (CP) and culture-negative (CN) groups in synovial fluid.

DISCUSSION

This study aimed to use *GAPDH* gene as an internal control for the validation of SF clinical samples. The process for developing internal controls in clinical studies is based on the theory that housekeeping genes have constant expression levels in all samples (Zhang et al., 2005). Inflammation in the joint area can develop into degenerative inflammation or infectious inflammation caused by aging or autoimmune disease. Then, *GAPDH* increases due to an increase in inflammation-related white blood cells and exfoliative cells (Cheon, 2002; Zainuddin et al., 2010). Therefore, housekeeping genes can be used as an internal control in molecular diagnostic techniques (Nazet et al., 2019; Ghasemi et al., 2017) for the normalization and validation of clinical samples. Several studies have investigated housekeeping genes, such as β-actin (*ACTB*) (Kim et al., 2012; Blaschke et al., 2000), *B2M* (Zhang et al., 2002), *RPL32* (Zhang et al., 2005), *RPLP0* (Nazet et al., 2019), *EEF1A1* (Nazet et al., 2019), *TBP* (Nazet et al., 2019; Zhang et al., 2005), *PPIB* (Nazet et al., 2019), *GAPDH* (Nazet et al., 2019; Zhang et al., 2002;

Zhang et al., 2005), and 18S rRNA (Nazet et al., 2019), with *GAPDH* being the most widely used (Nazet et al., 2019; Nakasa et al., 2008; Kanbe et al., 2002; Xiong et al., 2018; Kasama et al., 2000; Fang et al., 2018). In this study, primers and probes were designed for *GAPDH* to develop a new internal control *GAPDH* biomarker for SF samples. To detect *GAPDH* gene at this process of development, we designed new modified primers and probe that differed from the known *GAPDH* primers.

To confirm the specificity of the *GAPDH* gene set designed in this study, the *GAPDH* plasmid gene synthesized by the Macrogen Institute (Seoul, Republic of Korea) was used as a positive control. The cross-reactivity against the causative bacteria and fungi that cause PJIs was confirmed as a negative control. Subsequently, as a result of the cross-reactivity experiment, we found that amplification occurred only from the synthesized *GAPDH* plasmid (Fig. 2 and Table 4). To determine the sensitivity, 10-fold serial dilutions were performed. The minimum detection limit satisfying the *GAPDH* cut-off 30 criteria was 9.66×10^3 copies/μL (mean Ct value 27.23, standard deviation 0.06) (Table 3 and Fig. 1).

In a total of 113 samples collected from patients at the Mokdong Himchan Hospital and Incheon Himchan Hospital, 4 were classified as PJI and 109 as non-PJI based on the presence or absence of infection. Additionally, the microbiology culture tests conducted using the VITEK2 system found 11 CP and 102 CN cases (Fig. 4 and Table 8). Microorganism identification revealed that all 11 CP samples (100%, 11/11) were *GAPDH*-positive. Of the CN samples, 58 (56.9%, 58/102) were *GAPDH*-positive, and 44 (43.1%, 44/102) were *GAPDH*-negative. Specifically, the bacterial species identified in the 11 CP samples were *S. aureus* (2 cases), *S. epidermidis* (2 cases), *S. dysagalactiae* (2 cases), *E. coli* (2 cases), *S. marcescens* (1 case), *S. anginosus* (1 case), and *K. aerogenes* (1 case) (Table 5). Furthermore, the microbial identification in this study showed similar results as those in a previous study (Rho et al., 2020).

Additionally, the *P*-value and AUC value for the PJI and non-PJI groups and CP and CN groups for the Ct values of the GAPDH biomarker were AUC 0.8257 ($P = 0.0322$) and AUC 0.8975 ($P < 0.0001$), respectively (Fig. 5). In general, the expression of housekeeping genes used as internal controls in SF was constant (Kanbe et al., 2002). However, in the study, certain samples were not amplified by internal reference control (GAPDH) in SF, and these samples were excluded from data analysis (Fang et al., 2018). Therefore, in our study, we designed the use of *GAPDH* gene as an internal control in and performed qPCR based on it. Further study will be conducted on the 69 *GAPDH*-positive cases (61.0%, 69/113). Our findings indicate that clinical SF samples could be validated using the developed *GAPDH* biomarker prior to the identification of causative bacteria and fungi.

When bacteria or fungi are detected, false positives may occur not only due to infection but also due to contamination by skin flora during sample collection, so these should be excluded. Therefore, to detect prosthetic joint infections (PJIs), the new detection kit of PJI pathogen is necessary, and normalization and validation processes of clinical samples are required.

ACKNOWLEDGEMENT

This work was supported by Busan Techno Park by the Korea government (Busan).

CONFLICT OF INTEREST

There are no conflicts of interest (both financial and personal) to declare.

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https://doi.org/10.15616/BSL.2023.29.4.220
Cite this article as: Lee J, Baek E, Ahn H, Park Y, Kim G, Lim S, Lee S, Kim S. Validation of Synovial Fluid Clinical Samples for Molecular Detection of Pathogens Causing Prosthetic Joint Infection Using GAPDH Housekeeping Gene as Internal Control. Biomedical Science Letters. 2023. 29: 220-230.