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Identification of Bacterial Agents from Blood Samples Based on Phenotypic and Genotypic Methods in Kermanshah, West of Iran

İran'ın Batısı Kirmanşah'ta Fenotipik ve Genotipik Yöntemlere Dayalı Kan Örneklerinden Bakteriyel Ajanların Tanımlanması

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Abstract

Introduction: Sepsis is a syndrome of host systemic inflammatory response and is caused by microbial pathogens invading the bloodstream. Quick and accurate diagnosis is a key factor in the treatment of sepsis before complications occur. Therefore, this study aimed to identify the bacterial agents of sepsis from blood culture using phenotypic and polymerase chain reaction (PCR) techniques.
Materials and Methods: A total of 287 blood culture bottles of suspected patients in Imam Reza Hospital were taken and transferred to the microbiology laboratory of the medical school. Isolates were identified by Microgen Kit and API 20 E kit. The PCR sequence analysis of the 16S rDNA gene was implemented for molecular detection of bacterial isolates.
Results: Of the 287 suspected sepsis samples, 231 were negative and 56 were positive. Among the positive samples, five were positive by PCR despite being negative in the culture. Among 56 isolates, 16 (28.57%) were Gram-negative bacteria and 40 (71.42%) were Gram-positive bacteria.
Conclusion: In this study, the result of PCR-based 16S rDNA gene analysis revealed high sensitivity compared with other methods of bacterial detection. Overall, the fast sampling and quick and accurate diagnosis reduce unnecessary prescription of antibiotics by physicians.
Keywords: Blood culture, bacterial isolates, phenotypic identification, genotypic identification, polymerase chain reaction

Öz

Giriş: Sepsis, kan dolaşımını istila eden mikrobiyal patojenlere bağlı olarak ortaya çıkan konakçı sistemik enflamatuvar yanıt sendromudur. Hızlı ve doğru tanı, sepsis tedavisinde komplikasyonlar oluşmadan önce anahtar rol oynar. Bu nedenle, bu çalışmada fenotipik yöntem ve polimeraz zincir reaksiyonu (PCR) tekniği kullanılarak kan kültüründen sepsisin bakteriyel ajanlarının belirlenmesi amaçlanmıştır.
Gereç ve Yöntem: İmam Rıza Hastanesi'ndeki sepsis şüphesi olan hastalardan toplam 287 adet kan kültürü şişesi alınarak tıp fakültesi mikrobiyoloji laboratuvarına nakledildi. İzolatlar, Microgen Kit ve API 20 E kiti ile tanımlandı. Bakteriyel izolatların moleküler tespiti için 16S rDNA geninin PCR sekans analizi uygulandı.
Bulgular: İki yüz seksen yedi şüpheli sepsis örneğinden 231'i negatif, 56'sı pozitif olarak belirlendi. Pozitif numunelerin beşinde kültürde üreme olmamasına rağmen PCR ile pozitiflik saptandı. Elli altı izolattan 16'sı (%28,57) Gram-olumsuz bakteri, 40'ı (%71,42) Gram-olumlu bakteri idi.
Sonuç: Bu çalışmada, PCR tabanlı 16S rDNA geni yöntemi diğer bakteri tespit yöntemlerine kıyasla yüksek duyarlılığa sahipti. Genel olarak, hızlı örnekleme ve hızlı ve doğru teşhis, gereksiz antibiyotik kullanımını azaltır.
Anahtar Kelimeler: Kan kültürü, bakteriyel izolatlar, fenotipik tanımlama, genotipik tanımlama, polimeraz zincir reaksiyonu

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Introduction

Bloodstream infection (BSI) is one of the most infectious diseases and medical emergencies, which is responsible for 10-15% of mortality in the absence of diagnosis and suitable treatment[1,2]. Sepsis refers to the invasion of microbial pathogens or their products, such as toxins, into the bloodstream, and is characterized by a systemic inflammatory response to infection[3,4].

One of the fundamental goals of a clinical microbiology laboratory is to rapidly and accurately identify bacterial pathogens involved in infectious processes, especially BSIs[5].

Approximately 2.3% of sepsis cases occur in hospitalized patients[6]. Given the growing resistance to nosocomial infections in medical centers, rapid diagnostics can reduce the use of antimicrobial agents, side effects, and antibiotic resistance[7].

The common diagnostic methods of bacterial infections are microscopic and phenotypic examination (Gram staining), serologic methods for bacterial antistructural antibodies, and antimicrobial susceptibility testing[8-10]. Blood culture is the standard method of detecting blood infections when live microorganisms are detected in the blood.

Several studies have shown that the risk of mortality from BSI can be life-threatening if only a single microbiological diagnosis is used and may result in inadequate treatment[2,11,12]. Each molecular test is designed for a specific microorganism and only recognizes the same microorganisms. Molecular tests are divided into quantitative and qualitative tests. Quantitative experiments determine the severity of the infection and measure the amounts of genetic materials of a microorganism (such as *Pseudomonas aeruginosa* and *Streptococcus agalactia*). Qualitative methods are designed to determine the presence of microorganisms (such as *Mycobacterium tuberculosis*) and the effectiveness of prescribed medications[8,13]. Nucleic acid-based tests can be used to study genes or gene mutations in resistant bacteria[14]. Molecular methods, in addition to microorganism identification, improved the determination of genotypes (determining the characteristics of the strains) of important medical microorganisms[15]. Innovation of methods such as multiplex polymerase chain reaction (PCR) and reverse-transcriptase-PCR reduced the cost of molecular identification. As their role in the identification of microorganisms has increased, the management of infectious diseases is more focused on[10,15]. Moreover, molecular methods can identify hard-growing bacteria and viruses[15]. Generally, antimicrobial selective treatment for sepsis is not predetermined. Although in recent years antibiotics with a wide range of effects are available, the mortality from sepsis remains high.

The obtained results for isolates in the blood culture study in Kermanshah city can help us identify emerging resistances in these common hospital pathogens. This can be important in controlling and providing effective treatment for infections[12]. Therefore, this study examined the value of sequencing diagnosis for PCR-based 16S rDNA gene analysis for the detection of isolated bacterial agents from inpatients in Kermanshah Hospitals in 2017-2018. The key to the treatment of sepsis is a quick and accurate diagnosis before complications occur. Therefore, this study aimed to identify the bacterial agents of sepsis from blood culture using phenotypic and PCR techniques.

Materials and Methods

Bacterial Isolates

A total of 287 blood culture bottles of patients suspected of sepsis in Imam Reza Hospital were taken and transferred to the microbiology laboratory of the medical school. Blood samples were cultured on blood agar (Merck, Germany) and eosin methylen-blue (EMB) agar medium (Merck, Germany). After three weeks, cultures were examined for bacterial phenotypic diagnosis. Microorganisms were detected using API and microgen kits (bioMérieux, Marcy-l'Etoile, France). Blood culture bottles were first cleaned and dried with Betadine 70% alcohol (Merck). Using a suitable syringe, 1-2 ml was removed from the blood culture bottles, and a drop of blood was cultivated on the EMB agar. After cultivation, plates were incubated at 37 °C. Then, microorganisms were identified API.

API kit: This kit (bioMérieux) was used to identify Gram-negative bacteria[16]. After culturing on the kits for 24 and 48 h, the samples were examined. The results were read according to the protocol of the manufacturer. First, saline solution was added to a colony of the bacteria and compared with the McFarland standard solution. Then, the bacteria were inoculated with a sterile pipette inside each API bar. Some wells changed color due to differences in pH, and some other produced end products that should be identified with reagents.

Microgen kit: According to the kit protocol (bioMérieux), 2-MacFarland solution was prepared from each bacterium and added 100 µl to any wells from the McFarland suspension. Well no. 12 (arginine) was filled with oil. The wells were placed at 37 °C for 24 h. After 24 h, first, one drop of VP1 reagents was added to well no. 8 and then added with one drop of VP2 reagents. The results were read after 15-30 min. Then, one drop of PYR reagents was added to well no. 2 and read after 5-10 min. After 24 h, the bacterial type was determined based on the changes in colors and software (Figure 1).

DNA extraction by the boiling method: Using a suitable syringe, 1-2 ml of blood from the blood culture was removed, and a linear blood drop was cultured on enrichment agar and differential

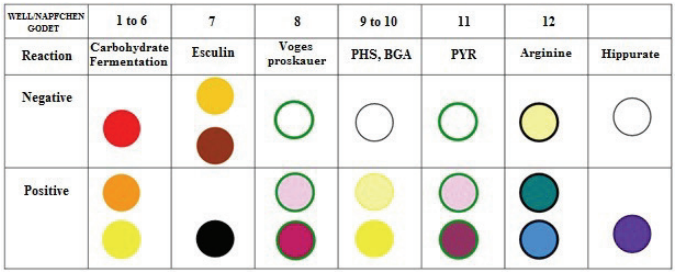

Figure 1. Commentary of microgen kit

culture medium of EMB agar (Merck) for 18-24 h. DNA was extracted using the boiling method. First, a certain amount of bacteria was dissolved in 500 µl of saline and centrifuged at 4000 rpm for 4 min, and the supernatant was discarded. This procedure was repeated twice, and microtubes were placed in a water bath at 100 °C for 30 min. Then, the microtube was placed at -20°C for three min and centrifuged again at 4000 rpm for 4 min. The supernatant was poured in a sterile microtube. The concentration and absorption of DNA were measured using NanoDrop One instrument (Thermo Fisher Scientific, Waltham, MA, USA). After obtaining arbitrary results in the absorption of 260-280, the DNA solution was stored at -20 °C.

Polymerase Chain Reaction Analysis and Electrophoresis

The appropriate primers of 16S rDNA were selected from a previous study[17] and were re-examined by online Oligocalc and OligoAnalyzer software v3.1 (Integrated DNA Technologies, Inc., Coralville, IA, USA). Primers were examined by BLAST in terms of complement or supplementation with the human genome. After confirmation, the sequences were designed and sent to Bioneer (South Korea). Primers were diluted according to the manufacturer's instruction (TAG, Copenhagen A/S, Denmark). To prepare this concentration, 5 µl from each primer (100 pmol) was removed and mixed with 45 µl of deionized water to rich 10 pmol of each primer and stored at -20 °C as a working vial. The settings and work schedules for 16S rDNA primers are listed in Table 1. The amplification process was programmed in a thermal cycler (MyCycler Thermal Cycler, Bio-Rad Laboratories Inc., CA, USA). Electrophoresis was performed after PCR to confirm the results. To perform electrophoresis, 1.5% agarose gel was prepared and samples were analyzed. The gel was placed in the gel documentation system (Bio-Rad Laboratories Inc.), and the result was recorded. Finally, the PCR product was sent to Bioneer (South Korea) for sequencing. The obtained results were analyzed using nucleotide sequence-based simulator software (BLAST n) and compared with other similar cases in the US Gene Bank, and the genus or species of the sepsis-causing bacteria were determined.

Statistical Analysis

The obtained results and data were entered into Excel software. Then, standard deviation, means, frequency, and percentages were calculated using IBM Statistical Package for the Social Sciences statistics version 20 (IBM Corp., Armonk, NY, USA).

Results

A total of 287 suspected sepsis samples were taken from 150 men and 137 women (Figure 2). The highest number of positive samples was taken from the internal ward, and the lowest number was from the critical care unit and orthopedic wards with one positive sample. The number of samples in different hospital wards is shown in Figure 3. In this study, of 56 isolates, 16 (28.57%) were Gram-negative, 40 (71.42%) were Gram-positive, and five were reported as negative in hospital records but identified as positive in our study (Table 2). In this study, the isolates in positive cultures with the highest number were *Staphylococcus hominis* (28.57%) and *S. aureus* (25%). The frequencies of *Escherichia coli* and *Klebsiella pneumoniae* in positive samples were 10.71% and 1.78%, respectively (Table 2 and Table 3). The PCR results showed that of 287 suspected sepsis samples, 231 were negative and 56 were positive. However, of 231 negative samples, the results of five samples were detected as positive by PCR (Figure 4).

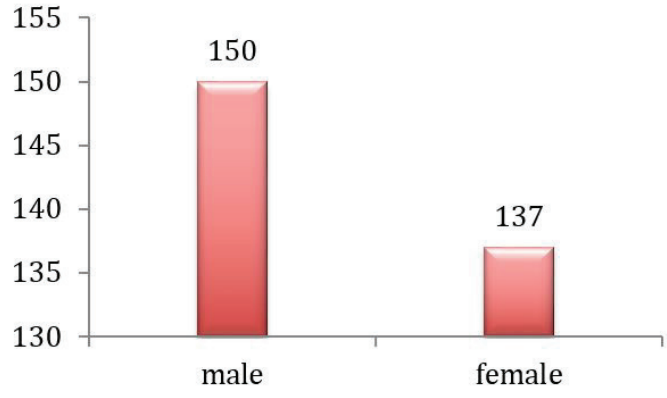

Figure 2. Frequency by sex

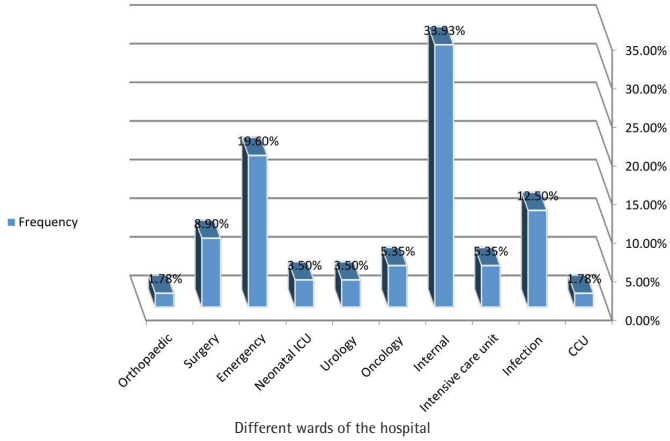

Figure 3. Frequency based on hospital wards
ICU: Intensive care unit, CCU: Critical care unit

Table 1. Primers sequences of 16S rDNA

Primers	Forward sequence	Reverse sequence	Expected size (pb)	Annealing temperature (°C)	Reference
16S rDNA	AACTGGAGGAAGGTGGGGAT	AGGAGGTGATCCAACCGCA	371	52	[19]

Table 2. Bacterial isolates collected in this study

Isolate segregations		Sex		Total
Name	Unit	Female	Male	
Escherichia coli	Count	3	3	6
	%	5.35	5.35	10.71
Achromobacter spp.	Count	3	0	3
	%	5.35	0	5.35
Citrobacter spp.	Count	0	4	4
	%	0	2.7	7.14
Klebsiella pneumoniae	Count	1	0	1
	%	1.78	0	1.78
Staphylococcus haemolyticus	Count	1	0	1
	%	1.78	0	1.78
Staphylococcus hominis	Count	6	10	16
	%	10.71	17.85	28.57
Staphylococcus saprophgticusyticus	Count	3	2	5
	%	5.35	3.57	8.92
Staphylococcus schleiferi	Count	0	1	1
	%	0	1.78	1.78
Staphylococcus spp.	Count	0	1	1
	%	0	1.78	1.78
Serratia spp.	Count	2	2	4
	%	3.57	3.57	7.14
Staphylococcus aureus	Count	8	6	14
	%	14.28	10.71	25
Total	Count	27	29	56
	%	48.21	51.78	100

Table 3. Comparison of diagnostic isolates of our study with those in the hospital

Number of isolates	Diagnostic bacteria of this study	Diagnostic bacteria in the hospital
4	*Escherichia coli*	*Escherichia coli*
1	*Citrobacter* spp.	*Escherichia coli*
3	*Citrobacter spp.*	*Citrobacter* spp.
12	*S. aureus*	*S. aureus*
2	*S. aureus*	*Staphylococcus* spp.
2	*Achromobacter* spp.	*Alcaligenes spp.*
3	*S. saprophyticus*	*Staphylococcus* spp.
1	*S. haemolyticus*	*Staphylococcus* spp.
12	*S. hominis*	*Staphylococcus* spp.
1	*S. saprophyticus*	CNS*
1	*S. hominis*	CNS
1	*Klebsiella pneumoniae*	*Citrobacter* spp.
4	*Serratia* spp.	*Serratia* spp.
2	*S. hominis*	*Enterococcus* spp.
1	*Staphylococcus* spp.	*Staphylococcus* spp.
1	*S. schleferi*	*Staphylococcus* spp.
1	*S. hominis*	Negative
1	*S. saprophyticus*	Negative
1	*Achromobacter* spp.	Negative
2	Escherichia coli	Negative

CNS: Central nervous system

Discussion

In this study, the isolates in positive cultures with the highest number were *S. hominis* (28.57%) and *S. aureus* (25%). The results of a systematic study and meta-analysis in Australia of more than 4,000 positive blood cultures collected from 13 countries showed that the most common causes of sepsis were *Klebsiella* spp. and *S. aureus*[4]. In the study by Sharifi Yazdi et al.[18] (2013), of 216 samples of sepsis, 55 (25.46%) were positive, whereas in our study, of 287 blood culture samples, 56 (19.51%) were positive samples and were consistent with our research. Among the positive samples in our study, in which the hospital conducted phenotypic and culture methods, of a total of 51 positive culture samples, just two samples of *S. coagulase* (3.92%) were reported as negative. In our study, the frequency of *E. coli* was 10.71%; *S. aureus,* 25%; and *K. pneumoniae,* 1.78%, in positive samples; both studies are consistent only in the frequency of *S. aureus*. Blood cultures are reported to be positive in 30-60% of sepsis cases and 80-60% of patients with septic shock. Gram-negative bacteria were found in 2.3% of the cases, Gram-positive bacteria in 10-20%, and fungi in 2-5% of the cases[3,19].

In the study by Ghoreyshi et al.[20] in 2007 in Tabriz, of their 210 positive samples, 12 types of bacteria have been isolated, with coagulase negative *Staphylococcus* as the most common. However, in our study, *S. hominis* was the most frequent. In their study, the most commonly identified microorganisms were *K. pneumoniae*, *E. coli*, and *S. hominis*, which is consistent with our study only in terms of the high frequency of *S. hominis* in positive cultures. Najeeb et al.[21] (2012) reported that of 130 positive blood cultures, Gram-negative bacteria were found in 71 (54.6%) and Gram-positive bacteria in 59 (45.4%) of the blood cultures. Contrary to our study that the frequencies of Gram-negative and Gram-positive bacteria were different, in this study, the frequencies were not much different. *S. aureus*

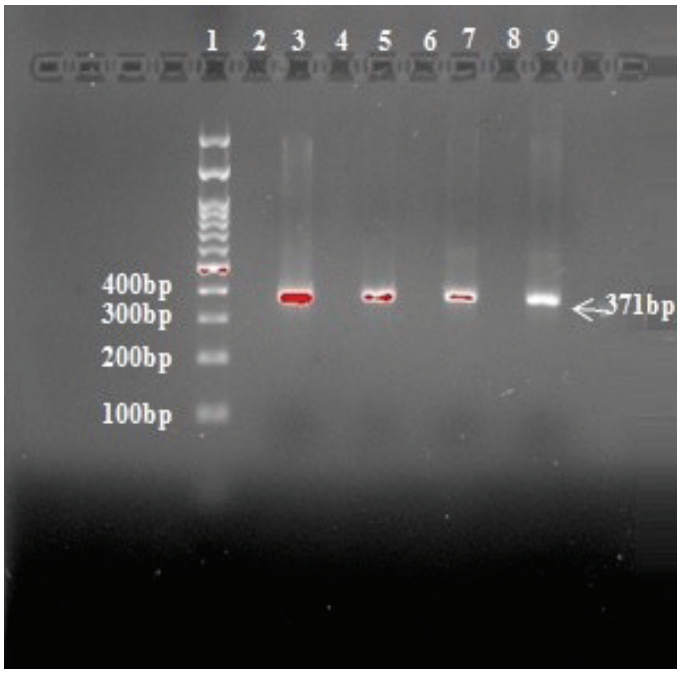

Figure 4. PCR results (Well 1, LADER; well 2, negative control; wells 3, positive control; wells 5, 7, and 9, positive samples)

PCR: Polymerase chain reaction

had the highest detection frequency, with 35 (26.9%) samples, followed by *E. coli* that was found in 30 (23.1%) samples. However, in our study, the frequency of *S. aureus* was 25% and that of *E. coli* was 10.71%. In the study by Heydarian et al.[22] in Mashhad, among 183 sepsis samples, the blood culture of 93 (50.8%) samples were positive, its frequency in our study was 19.51%, and *S. aureus* was reported as the most common cause of sepsis, which is consistent with our study. Pradipta et al.[23] conducted a study in Indonesia in 2015 and reported that 76 (39.5%) positive cultures were obtained from 192 patients, and *K. pneumoniae*, *E. coli*, and *S. hominis* were the isolates with the highest frequency. In our study, the frequencies of *E. coli* and *K. pneumoniae* were 10.71% and 1.78%, respectively. Liu et al.[24] compared 16S rDNA PCR with blood culture for the diagnosis of bacterial sepsis in infants and investigated the relationship between known factors, clinical signs, laboratory parameters, and diagnosis of sepsis. Approximately 706 infants suspected of sepsis were evaluated. The number of positive cultures and PCR-positive cases were 95 (13.5%) and 123 (17.4%), respectively. Compared with blood culture, PCR-based diagnosis of bacterial sepsis showed 100% sensitivity, 77.2% positive-predictive value, and 100% negative-predictive value. The most common bacterial causes were *Klebsiella* (43.7%) and *S. coagulase* negative (40.6%) which, in our study, had a frequency of 1.78%, which is different from our results. Different frequencies can be affected by the difference in time and geographical locations.

The 16S rDNA PCR has increased sensitivity and speed in diagnosing pathogens. It also shortens antibiotic durations, but blood culture is currently irreplaceable because the isolation of pure bacteria is necessary to test the susceptibility to antimicrobial drugs[24]. Jordan and Durso[25] compared PCR with routine blood culture. For this purpose, blood samples of 548 patients suspected of sepsis and admitted to the neonatal intensive care unit and 16S rDNA gene were used for diagnosing by PCR. The positivity rates using culture and PCR were 25 (4.6%) and 27 (4.9%) respectively. PCR had a sensitivity of 96.9%, specificity of 99.4%, positive-predictive value of 88.9%, and negative-predictive value of 99.8%.

In summary, the results showed that this PCR-based approach requires 9 h and 200 µl of blood and correlates well with normal blood culture results for infants suspected of bacterial sepsis[25]. Sepsis screening using a molecular method is less costly and time consuming than with culture analysis. In the study by Saderi et al.[26] in 2006 in Tehran, of 5116 suspicious cases, 912 (17.8%) were positive for culture, which is consistent with our study in terms of the frequency of positive cultures. The highest frequency of isolates was related to *P. aeruginosa* (58.4%), which was not detected in the positive cultures in our study. In 2002, a study in the United States analyzed 82569 positive blood culture samples, and the frequency of *S. coagulase* negative was 42%; *S. aureus,* 16.5%; *E. coli,* 7.2%; and *K. pneumoniae,* 3.6%[27]. In 2004, a study in India showed that the most common isolates of positive cultures were *S. aureus* with 13.8% and *K. pneumoniae* with 15%, and these findings were different from those in our study[28]. In the study by Rafati et al.[29] in 2013 in Sari, of 100 samples of sepsis suspected, 20 (20%) were positive by culture, which is consistent with our result (19.51%). The most common isolates were *S. aureus* (35%), *E. coli,* and *K. pneumoniae* (20%).

Study Limitations

(1) Low taxonomical resolution in 16SNGS reads.

(2) Costly laboratory setup and reagents.

(3) Lack of sample trial for repeat testing and lack of validation techniques.

(3) The 16S rDNA sequence is highly conserved and provides genetic information, but some species could not be identified in our study.

(4) High costs and requirements for great technical skill and lack of user-friendly comparative sequencing analysis software and validated databases.

(5) Not used beyond large and reference laboratories because of technical and cost considerations.

Conclusion

In this study, the result of PCR-based 16S rDNA gene analysis revealed that this method has high sensitivity compared with other methods for the detection of bacterial agents. Overall, the short time available between sampling, diagnosis, and receiving more accurate results reduced unnecessary prescription of antibiotics by physicians. Therefore, reducing unnecessary antibiotics and preventing antibiotic resistance reduces the duration of hospitalization and avoids imposing additional costs on the public health system and government.

Ethics

Ethics Committee Approval: This work was approved by the Research Ethics Committee of the Kermanshah University of Medical Sciences, Kermanshah, Iran (code: IR.KUMS.REC.1397.156, date: 12.06.2018).

Informed Consent: Informed consent is not required for the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.H., A.F., M.D., J.S., S.P.T., P.M., Concept: A.H., J.S., A.A., B.I., P.M., Design: A.H., A.F., M.D., S.P.T., B.I., P.M., Data Collection or Processing: A.H., A.A., B.I., P.M., Analysis or Interpretation: A.H., A.A., B.I., P.M., Literature Search: A.H., A.F., M.D., J.S., S.P.T., P.M., Writing: A.H., A.F., M.D., J.S., S.P.T., A.A., P.M.

Conflict of Interest: No conflict of interest was declared by the authors.

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