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Original Article

Analysis of Bacteria-derived Extracellular Vesicles in the Urine of Patients with Sepsis

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Abstract
Background *: Extracellular vesicles (EVs) are secreted by all bacteria, including those that cause sepsis, and are distributed throughout the bloodstream before getting excreted in the urine. The study aimed to analyze bacterial-derived extracellular vesicles in the urine of patients with sepsis and compare them with those in healthy controls.*
Methods *: The study included a total of 25 patients, comprising 9 cases with positive bacterial cultures in clinically significant specimens and 11 healthy controls. Urine samples, either midstream or collected via Foley catheter, were obtained before the initiation of antibiotic treatment. Extracellular vesicles were isolated from these samples using centrifugation. Metagenomic analysis was conducted on the isolated EV samples, focusing on bacterial 16S rDNA to identify the bacterial genera present.*
Results *: In all febrile patients with culture-positive cases, genetic material from multiple bacterial genera was detected through metagenomic analysis, although the specific bacteria identified in clinical cultures were not found in most cases. The bacterial distributions observed in the normal control group were markedly different from those in the febrile patients.*
Conclusions *: The findings suggest that bacterial components are likely entering the bloodstream from multiple sites within the body in the form of extracellular vesicles, both in septic and normal states. The distinct bacterial distributions observed in the urine of sepsis patients compared to healthy individuals require further investigation to understand the underlying mechanisms.*

Keywords: *Extracellular vesicles, Sepsis, Bacteria-derive*
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Introduction

Sepsis and its extreme manifestation, septic shock, are major clinical problems that kill millions of people around the world each year with a mortality of 35~55% in the case of septic shock[1]. While sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection, the treatment of sepsis usually demands fluid resuscitation, vasopressors, and other modalities beyond antibiotics.

The host response in sepsis is believed to be initiated and progressed by not only the microorganisms themselves but also their other cell components, including an endotoxin (lipopolysaccharide found in the bacterial outer cell membrane), peptidoglycan (a basic component of the bacterial cell wall) and their products including exotoxins. For this reason, the culprit organisms are not identified in up to 40-60% of patients with sepsis, even when the patients show full-blown septic manifestations[2].

On the other hand, the study of bacteria-derived extracellular vesicles (EVs) is a new research area that has recently been spotlighted in relation to the pathophysiology of infectious diseases and the pathogenesis of various noninfectious diseases. While EVs derived from Gram-negative bacteria were first discovered with the development of the electron microscope in the 1960s[3], recently, it was found that not only

Gram-negative bacteria but also Gram-positive bacteria such as *Staphylococcus aureus* secrete EVs[4].

EVs derived from bacteria are spherical vesicles with a size of 20-200 nm and have various biologically active substances such as proteins, lipids, and genetic material. As bacteria-derived EVs are released through the bacterial cell wall and the bacterial outer membrane, they contain components reflecting those structures, including lipopolysaccharides and peptidoglycans. Given that the properties of the bacteria-derived EVs overlap with several substances known to be involved in sepsis, it is likely that they are deeply involved in the pathogenesis of sepsis[5]. Moreover, bacteria-related substances, including endotoxins found in the body of sepsis patients, may actually exist in the form of EVs.

Bacteria-derived EVs are small-sized substances that are mostly excreted in the urine through the kidneys[6]. Therefore, it is expected that there will be a large amount of bacteria-derived EVs secreted from causative bacteria in the urine of patients with sepsis. Separation of these EVs from the urine of sepsis patients and analysis of the genetic material or proteins contained in the bacteria-derived EVs may reveal more precisely which bacteria and bacterial by-products are involved in sepsis.

This study was designed to analyze bacteria-derived EVs excreted in the urine of sepsis patients and evaluate if they are representative of the causative agents of sepsis.

Materials and methods

Participants

Patients with an acute fever over the age of 20 who visited the outpatient clinic or emergency room of Haeundae Paik Hospital of Inje University, were enrolled. People over the age of 20 who received a routine health check were selected as candidates for the healthy control group. We excluded patients with HIV infection or those who could not perform both methods to collect their urine: midstream urine or aseptic urine collection through a Foley catheter.

Sample collection

After explaining the study to the patients or their guardians and obtaining written consent, approximately 30-50 ml of midstream urine were collected in sterile containers. For the second method, the Foley catheter was inserted, and the urine was collected aseptically through the Foley catheter. Urine collection was performed before antibiotics were administered. The control group health checker collected urine at the time of the checkup. The collected urine was stored in a -70℃ freezer within 30 minutes of collection, and after being thawed, the EVs were isolated by centrifugation, and 16S metagenomics was performed at the time. This study was performed in accordance with the declaration of Helsinki. This study was approved by the Ethics Committee of Haeundae Paik Hospital of Inje University (No. 2013-051). Verbal informed consent was obtained from all the participants.

Extracellular vesicle (EV) isolation and DNA extraction from the urine samples

EVs in human urine were isolated using the differential centrifugation method, as described previously[7]. Briefly, urine samples were centrifuged at 10,000 x g for 10 min at 4˚C, and a 0.22 μg filter was used to eliminate bacteria and foreign particles from the supernatant. The DNA from the bacteria-derived EVs was extracted by boiling the filtrates for 40 min at 100˚C. DNA was then extracted using the DNeasy PowerSoil kit (QIAGEN, Germany).

Emulsion-based PCR for metagenomic sequencing

Sequencing was performed by Macrogen (Seoul, South Korea) using a GS-FLX Titanium Sequencer System (Roche, Basel, Switzerland). Briefly, genomic DNA was amplified from urine by the polymerase chain reaction (PCR). The method used a 16S rDNA fusion primer (27F, 5′-GAGTTTGATCMTGGCTCAG-3′ and the primer 518R, 5′-WTTACCGCGGCTGCTGG-3′) to amplify the V1–V3 region. GS-FLX titanium libraries were prepared using PCR products following the instructions provided in the GS-FLX Titanium Library Preparation Guide. EmPCR corresponding to clonal amplification of the purified libraries was performed using a GS-FLX titanium emPCR kit (454 Life Sciences, USA). Each sample was loaded onto one region of a 70 mm-75 mm PicoTiter plate (454 Life Sciences, USA) equipped with an 8-lane gasket for sequencing on a GS-FLX Titanium (454 Life Sciences, USA).

Analysis of the bacterial composition of the microbiota

Sequencing reads with high quality were retained by the quality score threshold (mean Phred score > 20 and read length > 300 bp). Operational Taxonomy Unit (OTU) was obtained using UCLUST and the taxonomic assignment was performed using QIIME and GreenGenes 8.15.13 database. Based on the similarity, all sequences were classified as follows: species, > 97% similarity; genus, > 94% similarity; family, > 90% similarity; order, > 85% similarity; 5 class, > 80% similarity; phylum, > 75% similarity.

Results

A total of 25 febrile patients were recruited over the course of 4 months from September 2013 to January 2014. There were 9 cases with positive cultures of bacteria in meaningful clinical specimens and 16 cases of culture-negative patients (Table 1).Eleven healthy controls were also evaluated for comparison to the febrile patients.

Table 1. Clinical and microbiological diagnosis of febrile patients.

Case No.	Age	Gender	Clinical diagnosis	Cultured bacteria	Specimens of the positive cultures	Other evidence used for disease diagnosis
1	54	M	Acute pyelonephritis	Escherichia coli	Blood, Urine	
2	61	M	Spondylitis	Staphylococcus epidermidis	Blood	
3	31	F	Unknown	Streptococcus agalactiae	Urine	
4	62	M	Acute pyelonephritis	Escherichia coli	Urine	
5	62	F	Acute pyelonephritis	Escherichia coli	Blood	
6	66	M	Pneumonia	Klebsiella pneumoniae	Sputum	
7	57	M	Endocarditis	Staphylococcus aureus	Blood	
8	72	M	Pneumonia	Pseudomonas aeruginosa, Staphylococcus aureus	Sputum	
9	45	M	Pacemaker infection	Staphylococcus aureus	Blood, Generator	
10	33	F	Kikuchi's disease			Lymph node pathologic findings
11	38	M	Kikuchi's disease			Lymph node pathologic findings
12	23	M	Kikuchi's disease			Lymph node pathologic findings
13	39	F	Tsutsugamushi' s disease			
14	52	F	Tsutsugamushi' s disease			Serology (+)
15	59	F	Tsutsugamushi' s disease			
16	54	M	Tsutsugamushi' s disease			Serology (+)
17	71	M	Sepsis			
18	67	M	Antibiotic-associated colitis			
19	91	M	Antibiotic-associated colitis			Stool Clostridium difficile toxin (+)
20	18	M	FUO			
21	70	F	Toxic shock syndrome			
22	69	F	Spondylitis			
23	53	M	Pneumonia			
24	37	M	Spondylitis			
25	35	F	Pneumonia			

Table 2 shows the results of the metagenomic analysis of the 16S rDNA from the bacteria-derived EVs analyzed in the urine of the febrile patients. In all the patients, including those whose clinical cultures were positive, the distribution of the bacteria-derived EVs of their urine was much more diverse than the bacteria grown from the clinical samples. In fact, bacterial proportions analyzed from the bacteria-derived EVs of urine matched the cultured bacteria in just a few patients at negligible percentages. Many other bacterial genera that are not known to be common causative bacteria including Caulobacteraceae, Novosphingobium, Bacillus, Propionibacterium, Rhizophila, Brevibacterium, and Sphingobium were generally distributed at higher proportions.

Table 2. Relative genus level proportion of bacterial EVs isolated from febrile patient urine.

No	Clinical diagnosis	Cultured bacteria	> 10%	5~10%	Proportion of cultured bacteria
1	Acute pyelonephritis	Escherichia coli	Caulobacteraceae (45.6%), Pseudomonas (11.3%)		Escherichia coli (0%)
2	Spondylitis	Staphylococcus epidermidis	Novosphingobium (28.1%)	Pseudomonas (9.5%), Propinonibacterium (5.2%)	Staphylococcus epidermidis (0.02%)
3	Unknown	Streptococcus agalactiae	Bacillus (19.5%), Propionibacterium (10.8%)	Pseudomonas (9.2%)	Streptococcus agalactiae (0%)
4	Acute pyelonephritis	Escherichia coli	Pseudomonas (11%)	Propionibacterium (6.7%) Enterobacter (6.4%)	Escherichia coli (0.02%)
5	Acute pyelonephritis	Escherichia coli	Caulobacteraceae (40%)	Propionibacterium (5.4%)	Escherichia coli (0.04%)
6	Pneumonia	Klebsiella pneumoniae	Pseudomonas (13.4%)		Klebsiella pneumoniae (0%)
7	Endocarditis	Staphylococcus aureus	Staphylococcus (61.1%)		Staphylococcus aureus (2.8%)
8	Pneumonia	Pseudomonas aeruginosa, Staphylococcus aureus	Propionibacterium (18.5%), Pseudomonas (10.4%)		Pseudomonas aeruginosa (1.5%), Staphylococcus aureus (0.02%)
9	Pacemaker infection	Staphylococcus aureus		Pseudomonas (8.9%)	Staphylococcus aureus (0.02%)
10	Kikuchi's disease		Xanthomonas (72.9%)		
11	Kikuchi's disease		Pseudomonas (14.9%)		
12	Kikuchi's disease		Pseudomonas (12.2%)		
13	Tsutsugamushi' s disease		Sphingobium (15.5%), Pseudomonas (12.9%)	Propionibacterium (7.4%)	
14	Tsutsugamushi' s disease		Staphylococcus (70.5%)		
15	Tsutsugamushi' s disease		Pseudomonas (91%)		
16	Tsutsugamushi' s disease		Xanthomonas (53.9%)	Rhizophila (8.9%), Pseudomonas (5%)	
17	Sepsis		Pseudomonas (17.5%)	Propionibacterium (7%)	
18	Antibiotic-associated colitis		Rhizophila (30.5%), Brevibacterium (20.5%), Xanthomonas (10.7%)		
19	Antibiotic-associated colitis		Ureaplasma (51.1%), Propionibacterium (11.9%)		
20	FUO		Staphylococcus (88.6%)		
21	Toxic shock syndrome		Staphylococcus (19%)	Pseudomonas (8.4%), Propionibacterium (5.4%), Enterobacter (5.3%)	
22	Spondylitis		Staphylococcus (13.2%)	Pseudomonas (7.5%), Bacillus (5.2%)	
23	Pneumonia		Propionibacterum (17.2%), Pseudomonas (9.4%)		
24	Spondylitis		Staphylococcus (43%)	Propionibacterium (6.5%)	
25	Pneumonia		Propionibacterum (11.7%), Pseudomonas (11.2%)		

Staphylococcus was the most common bacterial genus identified from the bacteria-derived EVs in the urine from a case of toxic shock syndrome and 2 cases of spondylitis. In these cases, although the causative bacteria were unable to be cultured, S. aureus is known to be the most common causative agent of the diseases. However, the Staphylococcus EVs of those patients could have originated from species other than S. aureus. For example, in the case of the endocarditis enrolled in this study, the proportion of S. aureus was just 2.8%, even though the total Staphylococcus proportion was 61.1%. Analysis of the bacterial genera to the species level was not attempted for the culture from the non-febrile patients.

As a result of the 16S metagenomic analysis of the bacteria-derived EVs in the urine of the healthy control group, the pattern of the bacterial distribution was shown to be significantly different from that of the febrile patients (Fig. 1).

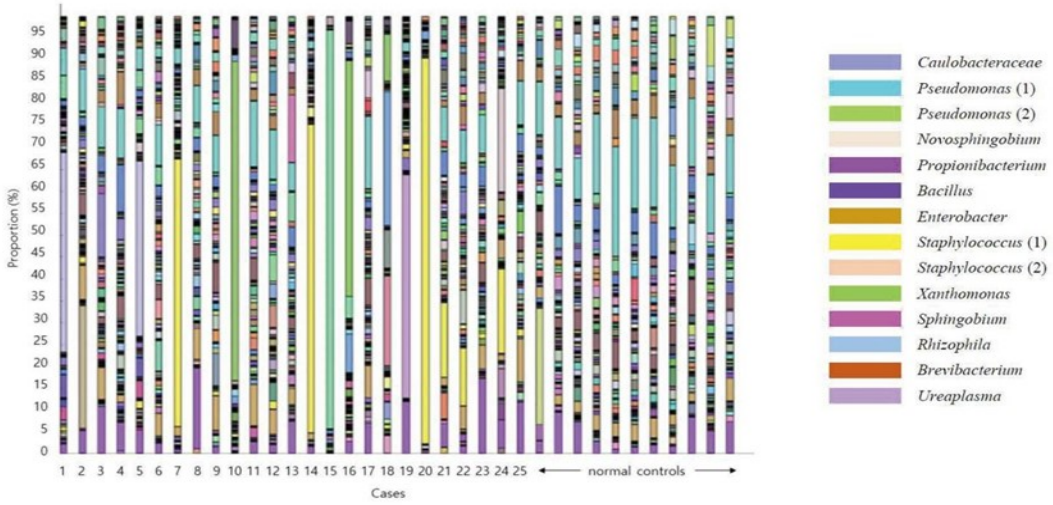

Figure 1.

Figure 1. Distribution of bacterial genera identified by 16S rDNA metagenomics of bacteria-derived extracellular vesicles in the urine of febrile patients and healthy people.

Discussion

The original purpose of this study was to develop a rapid and simple diagnostic method which helps identify the causative bacteria of sepsis by analyzing bacterial EVs in the urine of sepsis patients. However, the results of this study differed remarkably from our expectations. Evidence of the bacteria cultured in the clinical samples could not be found from the 16S metagenomic analysis of the bacteria-derived EVs in the urine of most cases. Even when they were detected in the bacterial EVs, the proportions were negligible. Instead, a variety of other bacteria prevailed as a repeated pattern in our analysis of the bacteria-derived EVs in the urine of sepsis patients, and most of the prevalent bacterial EVs were those not considered relevant causative agents in clinical practice. Rather unusual bacteria were also found in the urine of the healthy controls, although the distribution pattern differed from the febrile patients.

We initially were hesitant to publish these results as we could not explain the phenomena in a rational way. However, several articles with findings similar to ours have been released recently, leading us to decide to add our data to the discourse of this research area.

In a previous study, researchers conducted a 16s metagenomic analysis of blood from 75 febrile children in a West African country, Burkina Faso. They succeeded in matching blood cultured organisms in some cases; however, many unusual bacteria at the genus level were found in the 51 patients including Acinetobacter, Aerococcus, Shewanella, Rhodanobacter, Psillomonas, Flacklamia, Petrobacter, Geobacillus, Nocardioides, Filimonas, Propionispira, Lysobacter, Tissierella, eptotrichia, Paludimonas, Kineosphaera, Raoultella, Proteiniphilum, Amaricoccus, and Lutimonas[8]. The results were excluded from the analysis as irrelevant data, but they were too numerous to do so. A study analyzing 16S rDNA-based next-generation sequencing of blood and neutrophil-associated microbiomes in patients with severe acute pancreatitis found multi-

ple diverse bacterial genera in those patients regardless of their infection state[9]. In addition, bacterial genomes were found in healthy controls, whose distribution was different from those of severe acute pancreatitis patients. A similar study performed in patients with sepsis and healthy volunteers showed the presence of bacterial DNA both in septic and healthy participants with the bacterial diversity significantly higher in healthy volunteers[10]. Other numerous studies analyzing the bacterial genome in the blood of diverse participants have presented mixed results[11].

The most characteristic finding of this study is that diverse bacteria-derived EVs are present in the urine of both acute fever patients and healthy people. At the genus level, most of those bacteria are unusual microorganisms to be considered pathogens of sepsis. It is possible that contamination occurred during the testing process, especially in the case of those bacteria commonly found in the environment, such as dermatophytes, including *Propionibacterium* and *Bacillus,* or the so-called soil-bacteria like *Caulobacteraceae*, *Novosphingobium*, *Rhizophila*, *Brevibacterium*, and *Sphingobium*. However, considering the coincidence between the findings of this study and those of other previous publications demonstrating that the distribution pattern of the bacterial genome between sepsis patients and healthy people in blood and urine is different, we believe that the possibility of contamination is low. It would be more reasonable to assume that bacterial components come into our bodies and circulate throughout the body without our notice given the findings that the bacterial genomes are found even in the blood and urine of healthy people without evidence of infection.

In light of this evidence, we must carefully consider how bacterial components come into the bloodstream and are excreted in the urine. We have an abundant microbiota in our body that accounts for 10 times the number of cells as our own human cells and 150 times more bacterial genes than our own genome. This microbiota is particularly prevalent in the skin, oral cavity, gastrointestinal tract, and vagina[12]. Thus, the most likely origin of bacterial components, including EVs found in the blood and urine, is our normal flora. Because bacteria-derived EVs can be absorbed and bypass the GI tract mucosa[13], it would be reasonable to think that bacterial components often enter the bloodstream in the form of EVs rather than the bacteria themselves.

The reason why the genomes of bacteria-derived EVs in sepsis patients are different from the pattern of healthy people is not easy to postulate. Because polymicrobial infections are not common, it would be more reasonable to think that the bacterial components are also from sites inhabited by normal flora. Changes in the permeability of the mucosa induced by the septic process may permit the entrance of a variety of bacterial components, which would be blocked in normal conditions, causing the difference in the distribution of bacteria-derived EVs between sepsis patients and healthy people.

Our findings, together with previous studies demonstrating that abundant bacterial components from diverse bacterial genera circulate the body, suggest that the treatment paradigm for sepsis may need to be reconsidered. If cell-free bacterial components and EVs have a role in sepsis, antibiotics against cultured bacteria would be a little help as we have experienced in clinical settings. Anti-inflammatory medicine could help, but it would not significantly influence the circulating, abundant and diverse bacterial components. If we develop methods to filter out the bacterial components effectively from the bloodstream, it may offer an additional or possibly essential role in sepsis treatment.

A shortcoming of our study is that the analysis of the bacteria-derived EVs was not performed in blood samples simultaneously with the urine samples. Additional research needs to be done to see if the distribution of bacterial genomes in blood matches the pattern in the urine of sepsis patients. Another weak point of our study is that urine samples were collected just at one time before the start of antibiotics. Analysis of serial samples would give more information on the nature of the relationship between bacteria-derived EVs and sepsis. If experimental analysis with an animal sepsis model is performed, the data will also be much helpful to assess the influence of bacterial EVs in sepsis.

This work has special meaning in that the study performed 16S metagenomics for bacteria-derived EVs, not the bacteria themselves, in sepsis patients. Because the composition of the bacteria-derived EVs could be different from that of bacteria and the EVs are systemically distributed into many organs[13], the result of this study is expected to reflect the influence of the bacterial composition and its components on the pathologic process in sepsis more directly.

In conclusion, metagenomic analysis of 16S rDNA from bacteria-derived EVs in the urine of febrile patients showed the distribution of multiple bacterial genera that are generally uncommonly seen in relation to sepsis patients in clinical practice. Additional analysis of a healthy control group also produced a diverse bacterial distribution, the pattern of which was distinct from that of the febrile patients. Further studies, including simultaneous analysis of urine and blood samples, analysis at serial time points, and animal model studies, are needed to comprehensively explore

the nature of sepsis in relation to circulating bacterial EVs.

Data availability
All gene sequencing data analyzed during this study are available from the European Nucleotide Archive (ENA) database (https://www.ebi.ac.uk/ena/browser/home), accession number: PRJEB52409.

Competing Interests
The authors declare no competing interests.

Author Contributions
YKC contributed to the study concept, clinical data analysis, and manuscript writing. MR contributed to gene sequencing analysis. CK, JY, HS, TS, and YK contributed to EV isolation and gene sequencing. YJ and JK analyzed clinical data. SK contributed to study design, supervision, and manuscript review.

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