

## Comparison of Microbial Detection of Hemodialysis Water in Reasoner's 2A Agar (R2A) and Trypticase Soy Agar (TSA)

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The quality management of dialysis water used as dialysis fluid is important for patients exposed to large amounts of water. The treatment of dialysis water causes chemical and microbiological contamination. Dialysis water contaminated with bacteria causes various diseases and inflammatory reactions due to the inflow of toxins into the body. Consequently, the aim of this study was to understand the sensitivity of agar for the detection of bacteria in dialysis water, the seasonal characteristics of bacterial culture, and bacterial identification. In all, 420 samples of dialysis water collected from a hospital between September 2017 and August 2018 were cultured at clinical laboratories. The bacterial growth rate of R2A was 99 cases (23.5%), and that of TSA was 47 cases (11.1%). R2A was more sensitive than TSA for samples incubated above 1 CFU/ml in hemodialysis, and TSA was more sensitive than R2A for samples incubated above 50 CFU/ml. The morphological characteristics of the microorganisms were confirmed by gram staining 188 strains of 30 isolates from the specimens. In R2A, Gram-positive bacteria were isolated in 33.3% (n = 42), Gram-negative bacteria were isolated in 56.3% (n = 71), and fungal strains were isolated in 10.3% (n = 13). In TSA, Gram-positive bacteria were isolated in 33.8% (n = 21), Gram-negative bacteria were isolated in 64.5% (n = 40), and fungal strains were isolated in 1.6% (n = 1). In addition, seasonal distinctions were observed in microbial cultures.

**Key Words:** Healthcare-associated infection, HAI, Hemodialysis water, R2A, TSA

## INTRODUCTION

Healthcare-associated infection (HAI) includes nosocomial infections occurring in hospitals, out-of-hospital infections resulting from discharge, and outbreaks in the community (1). According to a study in the United States, HAI caused by multidrug-resistant bacteria infections occur in more than two million people each year. It was confirmed to account for 1% of all deaths (2). HAI includes surgical wound infections, bloodstream infections, urinary tract infections, and respiratory infections. Patients with significantly reduced immunity, such as the elderly, chronically ill, and organ transplant patients, are particularly vulnerable to HAI. Among the various treatments of patients, renal patients undergoing hemodialysis are treated with reduced immunity and are easily exposed to HAI, including injection needle accidents and multidrug-resistant bacteria, so infection control for hemodialysis is important (3, 4). According to the Korean Nephrological Association's

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**Table 1.** Maximum allowable levels of toxic chemicals and dialysis fluid electrolytes in dialysis water, Maximum allowable levels of trace elements in dialysis water\*

Contaminant	Maximum Concentration (mg/L)	Contaminant	Maximum Concentration (mg/L)	Contaminant	Maximum Concentration (mg/L)
Aluminum	0.01	Zinc	0.1	Beryllium	0.0004
Chloramines	0.1	Calcium	2 (0.1 mEq/L)	Cadmium	0.001
Free Chlorine	0.5	Magnesium	4 (0.3 mEq/L)	Chromium	0.014
Copper	0.1	Potassium	8 (0.2 mEq/L)	Mercury	0.0002
Fluoride	0.2	Sodium	70 (3.0 mEq/L)	Selenium	0.09
Lead	0.005	Antimony	0.006	Silver	0.005
Nitrate (as N)	2	Arsenic	0.005	Thallium	0.002
Sulfate	100	Barium	0.1		

\* Association for the Advancement of Medical Instrumentation (AAMI) 23500:2011

**Table 2.** Maximum allowable levels for total viable microbial count (TVC) and endotoxins in dialysis water \*

Contaminant	Maximum Allowable Level	Action Level
TVC <sup>†</sup>	< 100 CFU/ml	50 CFU/ml
endotoxins	0.25 EU/ml	0.125 EU/ml

\* Association for the Advancement of Medical Instrumentation (AAMI) 23500:2011

<sup>†</sup> TVC, Total Viable microbial Count

report “2018 Korea’s Renal Replacement Therapy,” the number of patients undergoing renal replacement therapy in Korea is 98,746, of whom 73,059 are undergoing hemodialysis. In particular, the number of renal failure patients per one million population was reported as 1,907. The number of people with renal failure is increasing every year, and the mortality rate is high, so many academics and the media are interested. Chronic kidney disease is generally associated with old age, diabetes, hypertension, obesity, and cardiovascular disease, and hemodialysis is a major treatment for these kidney diseases (5). Most patients with CKD receive hemodialysis three times a week, and the dialysis regimen is targeted to eliminate about two-thirds of urea during each treatment (6). Waste products, such as urea, diffuse through a thin membrane that separates the dialysate flowing in the opposite direction from the blood. Most bacteria and viruses present in dialysate have a high molecular weight and cannot pass through a thin membrane. However, toxins and the like have a small molecular weight, so they can pass through the membrane, requiring special attention in the use of the dialysate (7). The quality management of dialysis water used as dialysate is important for patients exposed to large amounts of water (8). Abnormalities in the water purification facility of dialysis water cause chemical and microbiological contamination, and dialysis water contaminated with bacteria causes not only inflammatory reactions but also various diseases due to the influx of toxins into the body (9). Fever from bacterial infection is caused by bacterial inflammatory substances and stimulates the secretion of interleukin-1 (IL-1) and cytokines by peripheral blood mononuclear cells (PBMC) (10). IL-1 causes an increase in C-reactive protein (CRP), and cytokine mediates the host response to infection and is involved in acute and chronic inflammation of bacterial ions (11). Therefore, even if there is no sign of fever, increased cytokine production due to the contamination of dialysis water causes chronic inflammatory conditions, resulting in dialysis-related amyloidosis, hypoalbuminemia, atherosclerosis, hypotension, and coma (12). In medical institutions that perform dialysis, HAI should be prevented through the regular monitoring of dialysis water. Detailed guidelines for managing dialysis water are provided by organizations such as the Association for the Advancement of Medical Instrumentation (AAMI), the American National Standards Institute (ANSI), the International Organization for Standardization (ISO), and the European Best Practice Guidelines (EBPG). According to the AAMI guidelines (13), the quality control of dialysis water includes a chemical test (Table 1) to confirm the presence of heavy metals and a microbiological test (Table 2) to confirm the presence or absence

of endotoxins and microorganisms. According to the AAMI guidelines, the acceptance criteria for dialysis water are less than 100 CFU/ml of bacteria and less than 0.25 EU/ml of endotoxins. In dialysis water, the action level (the concentration of bacterial contamination that must be taken before the maximum limit) is 50 CFU/ml of bacteria and 0.125 EU/ml of endotoxins, and corrective action must be taken quickly to drop below this level. As for the enforcement standards for each test, chemical tests should be conducted at least once a year, endotoxin tests must be conducted once a quarter, and microbial culture tests must be conducted once a month. For microbial culture tests, Trypticase Soy Agar (TSA) is recommended by the AAMI in the United States, and Reasoner's 2a Agar (R2A) is recommended by the International Organization for Standardization (ISO) (14). R2A and TSA studies reported that microbial cultures using R2A showed higher microbial detection compared to TSA cultures (12, 15). However, according to a study published in 2016, there was no significant distinction in microbial cultures using TSA and R2A (16). Looking at the above foreign cases, differences were confirmed in R2A and TSA. In addition, foreign research data did not include studies on environmental factors such as climate, season, and water quality in the country. In Korea, studies related to microbial culture tests and environmental factors for R2A and TSA in hemodialysis water are insufficient, and studies suitable for the domestic environment have been judged necessary to control infection and prevent HAI in hemodialysis water.

Therefore, this study aims to analyze the microbial culture and sensitivity of R2A, and TSA used in microbiological tests of hemodialysis water, identify seasonal microorganisms, and use them as basic data for improving hemodialysis infection control.

## MATERIALS AND METHODS

### Study sample

The hemodialysis water used in this study was a sample requested for microbial culture from September 2017 to August 2018, and 35 samples per month, a total of 420 samples were studied. According to the type of specimen, 396 cases of dialysis water and 24 cases of reverse osmosis water are classified.

### Sample Transport

The samples were collected in a sterile, endotoxin-free container, and immediately transported to the laboratory for testing within 30 minutes.

### Inoculation Media

Specimens requested from the laboratory were inoculated evenly on the surface of the medium by the spread plate method using a pipette in a TSA (Micromedia Co. Ltd., Korea) and an R2A (Asan Co. Ltd., Korea) medium, respectively. All tests were conducted in a biological safety cabinet (BSC class II), and the same specimen was repeated three times.

### Culture Methods

As the culture media, TSA (Micromedia Co. Ltd., Korea) and R2A (Asan Co. Ltd., Korea) were selected, and an oxygen culture environment was selected as the culture condition. Regarding the incubation period, the TSA medium was cultured at 37°C for 48 hours, and the R2A medium was cultured at 27°C for one week (Table 3).

**Table 3.** Cultivation techniques in dialysis water

Medium	Technique	Inoculation volume	Incubation Conditions
TSA	Spread Plate	1000uL	37°C, 48 hour
R2A			27°C, 7 days

## Gram Stain

The pure cultured independent colonies were smeared on slides, dried, and stained according to the manufacturer's standard usage guidelines using a Gram stain reagent (YD Diagnostics Co. Ltd., Korea). The staining and morphological characteristics of the microorganisms were observed using a microscope (x1,000). Microorganisms were identified by dividing them into gram-positive bacteria, gram-negative bacteria, and fungi through microscope checks.

## Statistical Analysis

All experimental results were expressed as mean and standard deviation through a total of three experiments, and the statistical analysis tested for the mean value and significance using SPSS Version 25.0 for the window software program. The significance test was performed at the  $p < 0.05$  level.

## RESULTS

### Comparing the number of dialysis water samples with bacterial culture by R2A and TSA

Of the total 420 hemodialysis water samples, 28 cases (6.7%) showed positive results for both R2A and TSA, and 71 cases (16.9%) were positive for R2A and negative for TSA. There were 19 samples (4.5%) that showed negative results in R2A and positive results in TSA, and 302 cases (71.9%) were confirmed as negative results in both R2A and TSA (Table 4, Fig. 1).

### Comparison of the number of samples of dialysis water with bacterial positive cultures by R2A and TSA

In confirming the positive rate of each medium in all samples, the microorganisms detected in R2A were higher than those detected in TSA, with 99 cases (23.5%) in R2A and 47 cases (11.1%) in TSA (Table 5).

**Table 4.** Comparing the number of dialysis water samples with bacterial culture by R2A and TSA

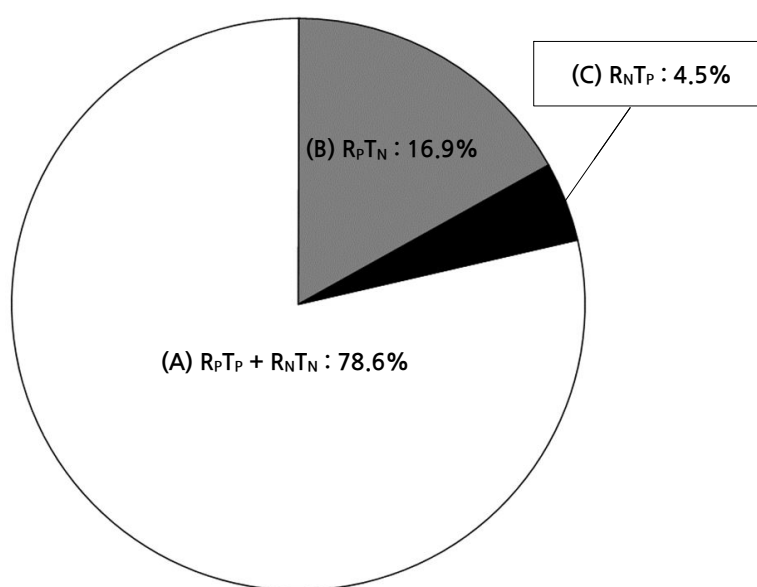
	Number of samples												Total (%)
	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	
R <sub>P</sub> T <sub>P</sub>	6	2	2	1	3	2	1	3	1	2	1	4	28 (6.7)
R <sub>P</sub> T <sub>N</sub>	8	8	4	3	14	2	3	3	13	2	6	5	71 (16.9)
R <sub>N</sub> T <sub>P</sub>	2	2	3	3	0	1	1	1	3	1	1	1	19 (4.5)
R <sub>N</sub> T <sub>N</sub>	19	23	26	28	18	30	30	28	18	30	27	25	302 (71.9)
Total	35	35	35	35	35	35	35	35	35	35	35	35	420 (100)

R<sub>P</sub>T<sub>P</sub>, Positive growth on R2A and TSA

R<sub>P</sub>T<sub>N</sub>, Positive growth on R2A and No growth on TSA

R<sub>N</sub>T<sub>P</sub>, No growth on R2A and Positive growth on TSA

R<sub>N</sub>T<sub>N</sub>, No growth on R2A and TSA



**Fig. 1.** Comparison of techniques for culturing dialysis water. (A) The positive growth of dialysis water and the negative growth of dialysis water were identified as 78.6% in both R2A and TSA. (B) The positive growth on R2A and negative growth of TSA were identified as 16.9% in dialysis water. (C) The positive growth on TSA and negative growth of R2A were identified as 4.5% in dialysis water.

**Table 5.** Comparison of the number of samples of dialysis water with bacterial positive cultures by R2A and TSA

Medium (TN)*	Number of samples												Total (%)
	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	
R2A (420)	14	10	6	4	17	4	4	6	14	4	7	9	99 (23.5)
TSA (420)	8	4	5	4	3	3	2	4	4	3	2	5	47 (11.1)

\* Total number of samples

**Table 6.** Comparison of bacteria growth on R2A and TSA media in identifying samples  $\geq 50$  CFU/ml

Medium (TN)*	Number of samples												Total (%)
	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	
R2A (420)	1	2	2	0	3	0	0	2	0	0	2	0	12 (2.8)
TSA (420)	3	2	3	0	1	1	0	4	2	2	0	0	18 (4.2)

\* Total number of samples

## Comparison of bacteria growth on R2A and TSA media in identifying samples $\geq 50$ CFU/ml

Among the 420 specimens, 12 (2.8%) in R2A and 18 (4.2%) in TSA were found to be cultured with more than 50 CFU/ml, and TSA was more sensitive than R2A (Table 6).

**Table 7.** Comparison of bacteria growth on R2A and TSA media in identifying samples <50 CFU/ml

Medium (TN)*	Number of samples												Total (%)
	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	
R2A (420)	13	8	4	4	14	4	4	4	14	4	5	9	87 (20.7)
TSA (420)	5	2	2	4	2	2	2	0	2	1	2	5	29 (6.9)

\* Total number of samples

## Comparison of bacteria growth on R2A and TSA media in identifying samples <50 CFU/ml

Among the 420 specimens, 87 (20.7%) in R2A and 29 (6.9%) in TSA were found to be cultured with less than 50 CFU/ml, and R2A was more sensitive than TSA (Table 7).

## Identification of heterotrophic bacteria isolated from dialysis water from September 2017 to August 2018

Of the 30 strains isolated from a total of 420 specimens, 188 strains were subcultured and studied. The isolated strains for each medium were identified as 126 in R2A and 62 in TSA, and the morphological characteristics of the microorganisms were confirmed through Gram staining.

In R2A, 42 Gram-positive strains (33.3%), 71 Gram-negative strains (56.3%), and yeast-like fungi accounted for 13 strains (10.3%). In TSA, 21 Gram-positive strains (33.8%), 40 Gram-negative strains (64.5%), and yeast-like fungi accounted for one strain (1.6%; Table 8).

## Seasonal distribution of bacterial populations isolated from dialysis water

The species of microorganisms separated from hemodialysis water were analyzed seasonally according to environmental factors, such as season and climate, in Korea (Table 9).

## Seasonal comparison of the number of samples of dialysis water with bacterial positive culture by R2A and TSA

Because of the seasonal analysis of the one-year study, the positive cases of microorganisms isolated from R2A were confirmed to be 24 cases in spring, 19 cases in summer, 30 cases in autumn, and 26 cases in winter. The positive cases of microorganisms isolated from TSA were 10 cases in spring, nine cases in summer, 17 cases in autumn, and 11 cases in winter. It was confirmed that the positive rate of microorganisms cultured in R2A medium was higher in all four seasons than that of microorganisms cultivated in TSA, and a high positive rate was confirmed in autumn in both R2A and TSA mediums, showing characteristic results (Table 10).

## DISCUSSION

The influx of microorganisms into the body is a risk factor that threatens human health by causing inflammatory reactions and various diseases. The rapid cultivation and identification of microorganisms is required for the treatment and prognosis

**Table 8.** Identification of heterotrophic bacteria isolated from dialysis water from September 2017 to August 2018

Gram stain	Genus/Species	Number of isolates	
		R2A	TSA
Gram Positive	<i>Staphylococcus epidermidis</i>	23	6
	<i>Staphylococcus hominis</i>	6	2
	<i>Kocuria rosea</i>	3	3
	<i>Staphylococcus capitis</i>	2	3
	<i>Staphylococcus warneri</i>	-	2
	<i>Granulicatella elegans</i>	-	2
	<i>Staphylococcus vitulinus</i>	-	1
	<i>Globicatella sanguinis</i>	-	1
	<i>Kocuria rhizophila</i>	-	1
	<i>Micrococcus luteus</i>	7	-
	<i>Kocuria kristinae</i>	1	-
	Total isolates	42	21
Gram Negative	<i>Sphingomonas paucimobilis</i>	27	9
	<i>Acinetobacter lwoffii</i>	12	3
	<i>Oligella ureolytica</i>	10	7
	<i>Roseomonas gilardii</i>	8	2
	<i>Ralstonia pickettii</i>	7	1
	<i>Burkholderia cepacia</i>	1	1
	<i>Pantoea</i> spp.	-	4
	<i>Achromobacter xylosoxidans</i>	-	4
	<i>Delftia acidovorans</i>	-	1
	<i>Bordetella bronchiseptica</i>	-	2
	<i>Comamonas testosteroni</i>	-	2
	<i>Aeromonas sobria</i>	-	1
	<i>Aeromonas salmonicida</i>	-	1
	<i>Moraxella</i> spp.	-	1
	<i>Brucella</i> spp.	-	1
	<i>Brevundimonas diminuta</i>	2	-
	<i>Methylobacterium</i> spp.	2	-
	<i>Cupriavidus pauculus</i>	2	-
	Total isolates	71	40
Yeast like fungus	<i>Candida sake</i>	13	1

of diseases. Consequently, various media have been developed, and there have been changes in the culture environment and conditions. In particular, the selection of the medium is the most important factor in increasing the detection rate of microorganisms. In this study, microbiological tests of hemodialysis water were performed using R2A and TSA to confirm the positive rate for each medium, the positive rate for each season, and the results of microbial identification. This contributed greatly to the prevention of medical-related infections and the management of artificial kidney center infections implemented in medical institutions. It is the first in the world to reflect climate change and seasonal specificity, and it is a meaningful study conducted over a long time period. The positive rates of microorganisms in hemodialysis water were confirmed in 99 cases (23.5%) in the R2A medium and 47 cases (11.1%) in the TSA medium. Therefore, the R2A

**Table 9.** Seasonal distribution of bacterial populations isolated from dialysis water

Medium	Spring	Summer	Fall	Winter
R2A	<i>Kocuria rosea</i> <i>Staphylococcus epidermidis</i>  <i>Acinetobacter lwoffii</i> <i>Burkholderia cepacia</i> <i>Cupriavidus pauculus</i> <i>Ralstonia pickettii</i> <i>Sphingomonas paucimobilis</i>  <i>Candida sake</i>	<i>Kocuria kristinae</i> <i>Kocuria rosea</i> <i>Micrococcus luteus</i>  <i>Staphylococcus capitis</i> <i>Acinetobacter lwoffii</i> <i>Cupriavidus pauculus</i> <i>Ralstonia pickettii</i> <i>Sphingomonas paucimobilis</i>  <i>Candida sake</i>	<i>Staphylococcus capitis</i> <i>Staphylococcus epidermidis</i> <i>Staphylococcus hominis</i>  <i>Acinetobacter lwoffii</i> <i>Oligella ureolytica</i> <i>Roseomonas gilardii</i> <i>Sphingomonas paucimobilis</i>  <i>Granulitella elegans</i> <i>Kocuria rosea</i> <i>Staphylococcus epidermidis</i> <i>Staphylococcus hominis</i> <i>Staphylococcus vitulinus</i> <i>Staphylococcus warneri</i>	<i>Staphylococcus epidermidis</i> <i>Staphylococcus hominis</i>  <i>Acinetobacter lwoffii</i> <i>Brevundimonas diminuta</i> <i>Oligella ureolytica</i> <i>Roseomonas gilardii</i> <i>Ralstonia pickettii</i> <i>Sphingomonas paucimobilis</i>  <i>Globicatella sanguinis</i> <i>Kocuria rosea</i> <i>Staphylococcus capitis</i>
TSA	<i>Staphylococcus capitis</i> <i>Staphylococcus epidermidis</i> <i>Staphylococcus warneri</i>  <i>Achromobacter xylosoxidans</i> <i>Aeromonas salmonicida</i> <i>Burkholderia cepacia</i> <i>Moraxella</i> spp. <i>Ralstonia pickettii</i> <i>Sphingomonas paucimobilis</i>	<i>Staphylococcus epidermidis</i> <i>Kocuria rhizophila</i>  <i>Acinetobacter lwoffii</i> <i>Bordetella bronchiseptica</i> <i>Comamonas testosteroni</i> <i>Sphingomonas paucimobilis</i>	<i>Staphylococcus epidermidis</i> <i>Staphylococcus hominis</i> <i>Staphylococcus vitulinus</i> <i>Staphylococcus warneri</i>  <i>Achromobacter xylosoxidans</i> <i>Brucella</i> spp. <i>Oligella ureolytica</i> <i>Pantoea</i> spp. <i>Roseomonas gilardii</i> <i>Sphingomonas paucimobilis</i>	<i>Globicatella sanguinis</i> <i>Kocuria rosea</i> <i>Staphylococcus capitis</i>  <i>Acinetobacter lwoffii</i> <i>Aeromonas sobria</i> <i>Delftia acidovorans</i> <i>Oligella ureolytica</i>  <i>Candida sake</i>

**Table 10.** Seasonal comparison of the number of samples of dialysis water with bacterial positive culture by R2A and TSA

Medium (Total No. of samples)	Number of samples				
	Spring	Summer	Fall	Winter	Total (%)
R2A (420)	24	19	30	26	99 (23.5)
TSA (420)	10	9	17	11	47 (11.1)

medium was found to be more sensitive than the TSA medium. In the Netherlands (15), in a study conducted on 229 samples of hemodialysis water and reverse osmosis water, the R2A medium showed a higher positive rate than the TSA medium, confirming results similar to this study. In Thailand (12), a study conducted on 143 samples of reverse osmosis water, also confirmed that the R2A medium was more sensitive than the TSA medium. The positive rate of bacteria counts of 50 CFU/mL or higher was found to be 2.8% in R2A and 4.2% in TSA in this study, which was higher than that of 1.5% in R2A and 1.3% in TSA, the results of the US study. When comparing the results with those of advanced countries, it is thought that a more active management of hemodialysis water is necessary. R2A is a low-nutrient agar used with lower incubation temperatures and longer incubation times (17). However, TSA is a universal medium containing two peptones to support the growth of various microorganisms (18). Hence, the identification and positive rates of microorganisms



cultured in the two media are considered different. The 188 strains of 30 species isolated in this study were classified into 33.5% Gram-positive microorganisms, 59.0% Gram-negative microorganisms, and 7.4% yeast-like fungi. *Aeromonas* sp., rarely cultured in TSA, is a bacterium that exists in fresh water and has been reported as a zoonotic pathogen that causes corneal inflammation in a recent domestic study (19). *Moraxella* sp. is also known as the causative agent of infectious diseases of otolaryngology (20). In Japan, bloodstream infections caused by *Methylobacterium* sp. have been reported in patients undergoing hemodialysis (21). Especially *Sphingomonas paucimobilis*, *Acinetobacter lwoffii*, and *Oligella ureolytica* showed high separation rates among Gram-negative microorganisms. In a Thai study, *Pseudomonas* spp. was 40%, *Moraxella* spp. 23%, *Acinetobacter* spp. 16%, *Staphylococcus* spp. 16%, *Alcaligenase* spp. 14%, Gram-negative rod 7%, *Corynebacterium* spp. 3%, *Micrococcus* spp. 3%, *Bacillus* spp. 1%, *Chromobacterium* spp. 1%, Gram-positive rod 1%, *Rhodococcus* spp. 1%, and *Streptococcus* spp. 1% was isolated, which was like this study in that the separation rate of Gram-negative microorganisms was high (12). The species of microorganisms isolated from hemodialysis water differed from season to season, and *Acinetobacter lwoffii* and *Sphingomonas paucimobilis* were identified as bacteria isolated throughout the year from R2A. *Sphingomonas paucimobilis* is an opportunistic pathogen that causes meningitis, sepsis, bacteremia, and peritonitis in people with reduced immunity (22). *Acinetobacter lwoffii* is a potential opportunistic pathogen in patients with impaired immune systems, and it has been identified as a cause of healthcare-associated infections (23). It is necessary to manage infections more effectively for hemodialysis water by monitoring bacteria that are separated year-round and bacteria that are separated by season. In foreign countries, there have been no studies on the detection of microorganisms in hemodialysis water related to season or climate. However, in Korea, the four seasons are distinct, and the temperature change varies greatly from season to season. The cultivation of microorganisms is closely related to the temperature and growth of bacteria, as well as the components of the medium and cultivation time. Therefore, if the R2A medium and the TSA medium are used together for the microbiological testing of hemodialysis water, it is believed that they can contribute to patient safety and public health safety infections.

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