

## **Original**

# **Investigation of a Membrane Filter Protocol for Direct Identification of Microorganisms in Dog Ear Discharge using MALDI-TOF MS**

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# Investigation of a Membrane Filter Protocol for Direct Identification of Microorganisms in Dog Ear Discharge using MALDI-TOF MS

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**Abstract:** Otitis externa is an ear disease that is present in 10-20% of dogs. Bacterial organisms are a common cause, and infection may cause severe and chronic inflammation. Thus, there is a need for early detection and identification of bacteria in ear discharge samples. In this study, 31 such samples were analyzed to identify microorganisms using MALDI-TOF MS with pretreatment using a membrane filtration protocol and a rapid BACpro II kit, compared to pretreatment with a rapid BACpro II kit alone. Reliable scores for *E. coli* and *E. faecalis* were obtained after inoculation of samples with  $1.0 \times 10^4$  CFU/ml using the membrane filter with the rapid BACpro II kit, with approximately 10-fold higher sensitivity compared to rapid BACpro pretreatment alone. Among 31 bacteria-positive colonies in the samples, MALDI-TOF MS and membrane filtration (0.80  $\mu$ m) with a rapid BACpro II kit was significantly more accurate compared to use of the kit alone (29 (82.9%) vs. 17 (48.6%) isolates identified,  $p < 0.001$ ). These results show that MALDI-TOF MS with a membrane filter and a rapid BACpro II kit is a quick and reliable method for bacterial identification in ear discharge samples.

**Keywords :** dog / ear discharge / membrane filtration / Rapid BACpro II kit / MALDI-TOF MS.

## INTRODUCTION

Otitis externa is the most common ear disease of dogs and may be present in 10% to 20% of the canine population (Angus 2004; Cole 2004). Common clinical signs of otitis externa are head shaking and scratching of the ears (Cole 2004). Numerous causative agents have been associated with otitis externa, with bacterial organisms being one of the most important. Several different species of bacteria have been isolated from dogs with otitis externa, with *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas* spp., *Proteus* spp., and *Escherichia coli* found to be most prevalent (Greene 2006; Scott 2001). It is important to note that the bacteria or yeasts associated with cases of otitis

externa are only opportunists and are not the primary pathogens (Rosser 2004; Scott 2001). Infection by microorganisms often causes severe and chronic inflammation. Antibiotics are mainly used for treatment of otitis infection, but inappropriate treatment may complicate the ear infection and result in increased antibiotic-resistant organisms. However, conventional systematic bacteria culture is limited by its low predictive value and time-consuming nature (Baba et al. 1981). Thus, a method that can identify bacteria with high sensitivity and specificity is needed for early detection.

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is a powerful tool for identification of pathogens, including bacteria. MALDI-TOF MS has revolutionized routine microbiology practice by decreasing the analysis time at different stages (DeCarolis 2014; Nomura 2015). As we have previously described (Sogawa 2011), the MALDI Biotyper system (Bruker

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Daltonik, GmbH, Bremen, Germany) enables rapid detection of bacteria in clinical samples by skipping cultivation on agar plates, and can be used for blood, urine, and peritoneal, synovial, and cerebrospinal fluids (Catry 2006; Kitagawa 2018; Oviaño 2018). However, MALDI-TOF MS requires high bacterial counts to provide reliable scores. In previous reports, the identification rate was high for *E. coli* (97.6–100.0%), but low for *E. faecalis* (60.0–66.7%) in urine specimens with  $\geq 10^5$  CFU/mL, showing rate variation among bacterial species (Ferreira 2010; Kim 2015). Furthermore, inoculation with at least  $1.0 \times 10^5$  CFU/mL was required to obtain reliable scores for *E. coli* and *E. faecalis* after preparation by rapid BACpro® in urine specimens (Maeda 2018). Most bacteria exhibit properties that make them adherent to positively charged polymer surfaces, a characteristic that has been utilized in wastewater treatment. Bacterial flocculation occurs through the copolymerization of polyallylamine and polystyrene, with bacteria being precipitated by magnesium chloride. Nittobo Medical has developed a method for collecting bacteria from positive blood culture bottles. This pretreatment protocol was initially marketed as the rapid BACpro®, and an improved version is now commercially available as the rapid BACpro II kit (Nittobo Medical Co., Tokyo, Japan). The minimum detectable sensitivity of the rapid BACpro II kit has been reported to be  $1.0 \times 10^5$  CFU/mL for *E. coli* and *E. faecalis* (Tsuchida, 2018).

Ear discharge collected using sterile cotton swabs is viscous and includes epithelial cells. Using MALDI-TOF MS with the rapid BACpro® and 0.30 µm filtration method (Sogawa, 2021), we successfully identified 14 intestinal Gram-negative bacteria (93.0%) and 55 Gram-positive cocci (87.3%) in perioperative drainage fluid from hepatobiliary-pancreatic surgery. Membrane filters with a pore size of 0.3 µm were used for filtration and sterilization, while those with a pore size of 0.8 µm were chosen as common filters for collecting yeasts and fungi and for reducing viscosity. These filters with pore diameters of 0.3 µm and 0.8 µm were used to collect microorganisms. In addition, the bacteria volume was small due to collection using sterile cotton swabs. To extract bacteria only in a solution in which a membrane filter was washed, a rapid BACpro kit was used concomitantly.

Scores were determined for the MALDI Biotyper system with pretreatment using a membrane filtration protocol and a rapid BACpro II kit, compared to pretreatment with a rapid BACpro II kit only for direct identification of bacteria in otitis externa. Through comparison with a conventional method for identification of microorganisms, we show that the MALDI Biotyper system with a membrane filtration protocol is more beneficial for clinical microbiology analysis, with advantages such as higher sensitivity, higher accuracy and rapid identification.

## MATERIALS AND METHODS

In this study, experiments were conducted based on the rapid BACpro® and 0.30 µm filtration method (Sogawa, 2021), with some modifications.

### Animals and samples

Ear discharge samples from 31 dogs (18 males, 13 females; age 0–10 years) brought to Maeda Veterinary Hospital between October 11, 2023 and February 29, 2024 were used in the study.

### Bacterial count

Bacterial counts were measured using the bioMérieux TEMPO® system (bioMérieux Japan Ltd. Tokyo, Japan).

### Conventional identification

Bacterial identification with the conventional method was performed using a MicroScan WalkAway plus system (Beckman Coulter, Brea, CA, USA). For the conventional culture, ear discharge samples were inoculated and spread on Nissui Plate Sheep Blood Agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) using a sterile plastic disposable loop (Eiken Chemical Co., Tokyo, Japan). Plates were incubated in an aerobic atmosphere at 37°C for 18 to 24 h. When bacterial growth was observed, the colonies on blood agar were counted and colonies from plates were identified using the MicroScan WalkAway plus system.

### 16S rRNA gene sequencing and sequence analysis

Total DNA of fresh colonies was prepared using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed as previously described (Okazaki 2009, Otsuka 2005) using primers 8UA (5'-AGA GTT TGA TC(A/C) TGG CTC AG-3') and 1485B (5'-TAC GGT TAC CTT GTT ACG AC-3'). Amplicons were purified and sequenced using primers 519A (5'-CAG C(A/C)G CCG CGG TAA T-3'), 519B (5'-ATT ACC GCG GC(G/T) GCT G-3'), 907A (5'-AAA CT(T/C)AAA(T/G)GA ATT GAC GG-3'), and 907B (5'-CCG TCA ATT C(A/G)TTT(A/G)A GTT T-3'). PCR products were sequenced via Sanger sequencing using a 3730XL DNA analyzer (Applied Biosystems). A homology search of 16S rRNA gene sequences was performed against sequences registered in GenBank/EMBL/DDBJ using BLAST. Final sequencing identifications of 16S rRNA was assigned according to Clinical and Laboratory Standards Institute (CLSI) interpretive criteria.

### Membrane filtration

Ear discharge samples were collected using a sterile cotton swab. Aliquots (1 mL) of sterile water were inoculated with an ear discharge sample and centrifuged at  $2,000 \times g$  for 30 sec. The supernatant was manually drawn into a 1 mL

syringe fitted with a 0.30- $\mu$ m or 0.80- $\mu$ m MF-Millipore membrane filter (Merck KGaA, Darmstadt, Germany). This procedure was completed within 2 min. Subsequently, each membrane was manually washed with 5 mL of rapid BACpro II kit reaction buffer 1 delivered using a 1-mL syringe with a 0.30- $\mu$ m or 0.80- $\mu$ m membrane quality monitor. This procedure was also completed within 2 min.

#### Rapid BACpro II kit

The ear discharge sample preparation was also performed using the rapid BACpro II kit (Nittobo Medical Co., Tokyo, Japan) (Ashizawa 2017). In brief, ear discharge solution (1 mL) was centrifuged at 2,000 $\times$ g for 30 sec. The solution processed by membrane filtration (5 mL) or ear discharge solution (1 mL) were then centrifuged at 15,000 $\times$ g for 10 min for bacteria collection. The pellet samples were sequentially combined with 10  $\mu$ L of reaction buffer 1, 100  $\mu$ L of reaction buffer 2, and 10  $\mu$ L of polymer suspension. The mixture was separated using a desktop centrifuge (Chibitan R, Merck Millipore, Billerica, MA, USA), at 2,000 $\times$ g for 30 sec and the supernatant was discarded. The resulting aggregate was resuspended in 1,000  $\mu$ L of 70% acetonitrile and the supernatant was separated by centrifugation at 2,000 $\times$ g for 30 sec. Finally, the obtained precipitate was resuspended in 30  $\mu$ L of 70% formic acid and 100  $\mu$ L of 100% acetonitrile, and the supernatant was separated by centrifugation at 2,000 $\times$ g for 60 sec.

#### Bacterial identification by MALDI-TOF MS

An  $\alpha$ -cyano-4-hydroxycinnamic acid matrix was prepared daily as a saturated solution in 50% acetonitrile and 2.5% trifluoroacetic acid. Subsequently, 1  $\mu$ L of the sample extract (prepared using either of the three approaches above) was spotted on a steel target plate (Bruker Daltonics) and allowed to dry. Next, 1  $\mu$ L of matrix solution was added and air dried. The target plate was then placed in the MALDI-TOF MS apparatus. To identify the isolates, MALDI-TOF MS (MALDI Biotyper, Bruker Daltonics) was performed on a MALDI Biotyper equipped with MBT Compass (version 4.1, Bruker Daltonics) for automatic acquisition of mass spectra in the linear positive mode within a range of 2 to 20 kDa. All MALDI-TOF MS measurements in the study were performed by laboratory technicians.

The MALDI Biotyper was periodically calibrated using the Bruker Daltonics bacterial test standard (*Escherichia coli* extracts containing RNase A and myoglobin). Automated analysis of raw spectral data was performed using MBT Compass (Bruker Daltonics) with a library of 10,833 spectra (version 9) and default settings. The whole process, from MALDI-TOF MS measurement to identification, was performed automatically without user intervention. After alignment, peaks with a mass-to-charge ratio difference <250 ppm were deemed identical. The peak lists generated were matched against the reference library using an integrated pattern matching algorithm in the software. Pattern-

matching results are expressed as scores from 0.0 to 3.0, with a score <1.7 not considered to give reliable identification and a score >2.0 indicating identification of a species (Sogawa 2011).

#### Limitation of detection range for MALDI-TOF MS

*E. coli* (ATCC 4157) and *Enterococcus faecalis* (*E. faecalis*) (ATCC 7080) strains were purchased from American Type Culture Collection (ATCC®). Pellets of these standard strains were dissolved and incubated at 37°C for 18 to 24 hours before inoculation on blood agar media (Nippon Becton Dickinson, Fukushima, Japan).

To determine the minimal bacterial concentration allowing reliable MALDI-TOF MS identification, aliquots of sterile water (50 mL) were inoculated with one strain of *E. coli* or *E. faecalis* at a bacterial count of  $1.0 \times 10^8$  colony-forming unit (CFU)/mL. Sequential dilutions were performed to achieve aliquots of each microorganism at bacterial counts of  $1.0 \times 10^6$ ,  $1.0 \times 10^5$ ,  $1.0 \times 10^4$ ,  $1.0 \times 10^3$  and  $1.0 \times 10^2$  CFU/mL. If necessary, aliquots were further diluted before spreading on a plate to obtain a countable number of colonies (50 to 500). Three 100- $\mu$ L aliquots of each dilution were plated onto blood agar plates. These plates were incubated at 37°C in an aerobic atmosphere and colonies were counted manually. The mean of three aliquots of each dilution was defined as the final count for the aliquot. Samples (10 mL) of each dilution were taken for preparation for MALDI-TOF MS, which was performed using the membrane filtration and rapid BACpro II kit method.

## RESULTS

#### Increase in MALDI-TOF MS scores by membrane filtration with a Rapid BACpro II kit

Reliable scores for *E. coli* and *E. faecalis* in ear discharge specimens were obtained by inoculation with  $1.0 \times 10^4$  CFU/mL after preparation with a membrane filter and rapid BACpro II kit (Table 1). Inoculation of at least  $1.0 \times 10^5$  CFU/mL is necessary for reliable identification of the bacterial population using the rapid BACpro II kit alone, whereas use of this kit with our newly established pretreatment method requires only  $1.0 \times 10^4$  CFU/mL. Thus, use of a membrane filter with the rapid BACpro II kit increases the sensitivity by about 10-fold for microorganism identification.

#### Ear discharge results of specimens using MALDI-TOF MS

Of 31 ear discharge specimens, growth of colonies was observed in 30 (96.8%) specimens: 25 with 1-colony morphology and 5 with 2-colony morphology (Table 2). One specimen did not grow in culture, and MALDI-TOF MS did not identify a significant protein profile. In specimens with 1-colony morphology, MALDI-TOF MS correctly identified 15 isolates (60.0%) using the rapid BACpro II kit alone, whereas MALDI-TOF MS with the rapid BACpro II kit identi-

**TABLE 1.** MALDI-TOF MS scores for bacteria count of *Escherichia coli* and *Enterococcus faecalis*.

Pretreatment protocol	Microorganism	MALDI-TOF MS score for bacteria count (CFU/mL)				
		$1.0 \times 10^6$	$1.0 \times 10^5$	$1.0 \times 10^4$	$1.0 \times 10^3$	$1.0 \times 10^2$
Membrane filtration (0.30 µm MF-Millipore membrane filter)	<i>E. coli</i>	2.432	2.413	1.984	1.723	1.622
	<i>E. faecalis</i>	2.315	2.117	1.815	1.704	1.616
Membrane filtration (0.80 µm MF-Millipore membrane filter)	<i>E. coli</i>	2.384	2.332	1.982	1.734	1.611
	<i>E. faecalis</i>	2.206	2.041	1.803	1.701	1.614
Rapid BACpro II kit	<i>E. coli</i>	2.401	2.392	1.974	1.733	1.631
	<i>E. faecalis</i>	2.301	2.105	1.822	1.721	1.602
Membrane filtration with Rapid BACpro II kit (0.30 µm MF-Millipore membrane filter)	<i>E. coli</i>	2.428	2.406	2.126	1.824	1.692
	<i>E. faecalis</i>	2.325	2.204	2.014	1.805	1.711
Membrane filtration with Rapid BACpro II kit (0.80 µm MF-Millipore membrane filter)	<i>E. coli</i>	2.392	2.336	2.110	1.912	1.842
	<i>E. faecalis</i>	2.261	2.244	2.128	1.934	1.892

**TABLE 2.** Comparison of ear discharge specimens analyzed by MALDI-TOF MS with three pretreatment methods.

Ear discharge culture (no. of isolates)	Rapid BACpro II kit (no. of isolates)	Membrane filtration (0.30 µm) with Rapid BACpro II kit (no. of isolates)	Membrane filtration (0.80 µm) with Rapid BACpro II kit (no. of isolates)
Growth of colonies (N=30)			
1-colony morphology (N=25)	Identification at the species level (15) No reliable identification (10) <sup>a</sup>	Identification at the species level (19) No reliable identification (6) <sup>b</sup>	Identification at the species level (21) No reliable identification (4) <sup>b</sup>
2-colony morphology (N=5)	No reliable identification (5) <sup>a</sup>	<i>B. cereus</i> and <i>S. schleiferi</i> <sup>a</sup> No reliable identification (4) <sup>a</sup>	<i>B. cereus</i> and <i>S. schleiferi</i> <sup>a</sup> <i>M. pachydermatis</i> and <i>S. schleiferi</i> <sup>a</sup> <i>P. aeruginosa</i> and <i>S. schleiferi</i> <sup>a</sup> No reliable identification (2) <sup>b</sup>
No growth (N=1)			

no.: number.

<sup>a</sup> Ear discharge specimens were cultured with  $<1.0 \times 10^5$  CFU/mL.<sup>b</sup> Ear discharge specimens were cultured with  $<1.0 \times 10^4$  CFU/mL.

fied 19 (76.0%) and 21 (84.0%) isolates after filtration with 0.30-µm and 0.80-µm membranes, respectively, showing significantly higher accuracy ( $p < 0.01$ ; chi-squared test). In the 10 cases in which identification was not possible with a rapid BACpro II kit alone, the bacterial count was  $\leq 1.0 \times 10^5$  CFU/mL, which was below the detection limit. In the 6 cases with 0.30-µm filtration and 4 cases with 0.80-µm filtration in which identification was not possible, the bacterial count was  $\leq 1.0 \times 10^4$  CFU/mL, which was also below the detection limit of this method. However, in 2 cases with 0.80-µm filtration, bacterial counts of  $\leq 1.0 \times 10^4$  CFU/mL were identified. The two species were *Corynebacterium diphtheriae* and *Malassezia pachydermatis*.

Consistent with these findings, 0.80-µm filtration with a rapid BACpro II kit (60.0%) had higher accuracy for bacterial identification compared to that with the rapid BACpro II kit alone (0.0%) and 0.30-µm filtration with a rapid BACpro II kit (20.0%) in specimens with 2-colony morphology ( $p < 0.001$ ; chi-squared test). In the 2-colony morphology samples in which both species were identified, the presence

of a mixed culture was recognized by MBT Compass. Thus, the 0.80-µm filtration protocol with a rapid BACpro II kit had improved accuracy in bacterial identification compared to use of the rapid BACpro II kit alone.

Next, we compared the accuracy of the three different pretreatments for microorganism identification (Table 3). With the conventional method, 35 isolates were identified, including one isolate in 25 specimens with 1-colony morphology, and two isolates in each of 5 specimens (2 isolates  $\times$  5 specimens = 10 isolates) with 2-colony morphology. The 35 isolates included 3 of *Bacillus cereus*, one of *C. diphtheriae*, two of *M. pachydermatis*, 15 of *Staphylococcus pseudintermedius*, 10 of *Staphylococcus schleiferi*, one of *Pasteurella multocida*, and 3 of *Pseudomonas aeruginosa*. These 35 strains were subjected to 16S rRNA analysis and the results were identical for all the strains when the bacterial counts were  $>1.0 \times 10^4$  CFU/mL. 1-colony morphology, MALDI-TOF MS correctly identified *S. pseudintermedius* (11/14), *S. schleiferi* (4/6) using the rapid BACpro II kit alone, in comparison with *B. cereus*

**TABLE 3.** Identification of 35 microorganisms by MALDI-TOF MS with different pretreatment methods.

16S rRNA method	Conventional method (no. of isolation)	rapid BACpro II kit (no. of isolation)	Membrane filter (0.30 µm) with rapid BACpro II kit (no. of isolation)	Membrane filter (0.80 µm) with rapid BACpro II kit (no. of isolation)
1-colony morphology (N=25)				
<i>Bacillus cereus</i> (1)	<i>Bacillus cereus</i> (1)	No reliable identification (1)	<i>Bacillus cereus</i> (1)	<i>Bacillus cereus</i> (1)
<i>Corynebacterium diphtheriae</i> (1)	<i>Corynebacterium diphtheriae</i> (1)	No reliable identification (1)	No reliable identification (1)	<i>Corynebacterium diphtheriae</i> (1)
<i>Malassezia pachydermatis</i> (1)	<i>Malassezia pachydermatis</i> (1)	No reliable identification (2)	No reliable identification (1)	<i>Malassezia pachydermatis</i> (1)
<i>Pasteurella multocida</i> (1)	<i>Pasteurella multocida</i> (1)	No reliable identification (1)	<i>Pasteurella multocida</i> (1)	<i>Pasteurella multocida</i> (1)
<i>Pseudomonas aeruginosa</i> (1)	<i>Pseudomonas aeruginosa</i> (1)	No reliable identification (1)	<i>Pseudomonas aeruginosa</i> (1)	<i>Pseudomonas aeruginosa</i> (1)
<i>Staphylococcus pseudintermedius</i> (14)	<i>Staphylococcus pseudintermedius</i> (14)	<i>Staphylococcus pseudintermedius</i> (11) No reliable identification (3)	<i>Staphylococcus pseudintermedius</i> (12) No reliable identification (2)	<i>Staphylococcus pseudintermedius</i> (12) No reliable identification (2)
<i>Staphylococcus schleiferi</i> (6)	<i>Staphylococcus schleiferi</i> (6)	<i>Staphylococcus schleiferi</i> (4) No reliable identification (2)	<i>Staphylococcus schleiferi</i> (4) No reliable identification (2)	<i>Staphylococcus schleiferi</i> (4) No reliable identification (2)
2-colony morphology (N=5)				
<i>B. cereus</i> and <i>S. schleiferi</i> (2)	<i>B. cereus</i> and <i>S. schleiferi</i> (2)	No reliable identification (2)	<i>B. cereus</i> and <i>S. schleiferi</i> (1) No reliable identification (1)	<i>B. cereus</i> and <i>S. schleiferi</i> (1) No reliable identification (1)
<i>M. pachydermatis</i> and <i>S. schleiferi</i> (1)	<i>M. pachydermatis</i> and <i>S. schleiferi</i> (1)	No reliable identification (1)	No reliable identification (1)	<i>M. pachydermatis</i> and <i>S. schleiferi</i> (1)
<i>P. aeruginosa</i> and <i>S. schleiferi</i> (1)	<i>P. aeruginosa</i> and <i>S. schleiferi</i> (1)	No reliable identification (1)	No reliable identification (1)	<i>P. aeruginosa</i> and <i>S. schleiferi</i> (1)
<i>P. aeruginosa</i> and <i>S. pseudintermedius</i> (1)	<i>P. aeruginosa</i> and <i>S. pseudintermedius</i> (1)	No reliable identification (1)	No reliable identification (1)	No reliable identification (1)
Total (30)	100.0% (30/30)	50.0% (15/30)	66.6% (20/30)	80.0% (24/30)

(1/1), *S. pseudintermedius* (12/14), *S. schleiferi* (4/6), *P. multocida* (1/1) and *P. aeruginosa* (1/1) with 0.30-µm membrane filtration with a rapid BACpro II kit; and *B. cereus* (1/1), *C. diphtheriae* (1/1), *M. pachydermatis* (1/1), *S. pseudintermedius* (12/14), *S. schleiferi* (4/6), *P. multocida* (1/1) and *P. aeruginosa* (1/1) with 0.80-µm membrane filtration with a rapid BACpro II kit. In 2-colony morphology, MALDI-TOF MS failed to identify all five samples using the rapid BACpro II kit only. In contrast, 0.30-µm membrane filtration with a rapid BACpro II kit correctly identified one sample, *B. cereus* and *S. schleiferi*, and 0.80-µm membrane filtration with a rapid BACpro II kit correctly identified three samples, *B. cereus* and *S. schleiferi*, *M. pachydermatis* and *S. schleiferi*, and *P. aeruginosa* and *S. schleiferi*. The three samples with 2-colony morphology were identifiable because the occupancy of each of the two species was 40-60%, and thus, the amounts of the two species were almost the same. Overall, among the 22 specimens with colony counts  $\geq 1.0 \times 10^4$  CFU/mL, microorganism identification by the conventional method coincided with the rapid BACpro II kit alone in 15 cases (68.2%), with 0.30-µm membrane filtration with the kit in 19 cases (86.4%), and with 0.80-µm membrane filtration with the kit in 22 cases (100.0%), with a significant difference between the three pretreatment methods ( $p < 0.01$ ; chi-squared test). Thus, the accuracy of microorganism identification using 0.80-µm membrane filtration with a rapid BACpro II kit is superior to using the kit alone and using 0.30-µm membrane filtration with the kit.

## DISCUSSION

The rapid BACpro II kit was developed to detect Gram-

positive and Gram-negative bacteria as well as yeasts from human blood cultures within a time period of 30 minutes (Tsuchida 2018). We found that reliable scores for *E. coli* and *E. faecalis* were obtained by inoculating a rapid BACpro® in drainage fluid and urine specimens with  $1.0 \times 10^5$  CFU/mL (Maeda 2018; Sogawa 2021). Additionally, reliable scores for *E. coli* and *E. faecalis* were achieved using the rapid BACpro® and 0.30 µm filtration method in drainage fluid specimens with  $1.0 \times 10^4$  CFU/mL (Sogawa, 2021). In this study, we showed that MALDI-TOF MS with a membrane filtration protocol can be used to identify microorganisms in canine ear discharge samples with high sensitivity and accuracy. An in-house membrane filtration method was developed to ensure high sensitivity and to enhance the identification rates of microorganisms in ear discharge samples.

In 1-colony morphology, the concordance rates of identification using MALDI-TOF MS with pretreatment with the rapid BACpro II kit and membrane filtration (0.8 µm) and the rapid BACpro II kit only were 92.0 and 60.0%, respectively (Table 3). We also observed an improvement in the identification rate when using the rapid BACpro II kit and membrane filtration (0.3 µm) with perioperative drainage fluids of hepatobiliary pancreatic surgery (Sogawa 2021). The *E. faecalis* detection sensitivity of MALDI-TOF MS using a rapid BACpro kit and membrane filtration (0.3 µm and 0.8 µm) was  $1.0 \times 10^4$  CFU/mL (Table 1). Thus, MALDI-TOF MS identification of bacteria using a rapid BACpro II kit and membrane filtration pretreatment is possible at a bacterial level  $\geq 1.0 \times 10^4$  CFU/mL.

Ear discharge is a thick and viscous exudate fluid, in which tissues with inflammation resulting from bacterial



infection are included. When membrane filters with pore diameters of 0.3 µm and 0.8 µm were used in pretreatment, viscosity was observed on the surface of filters with a pore diameter of 0.3 µm, but not on the surface of those with a diameter of 0.8 µm. The bacteria collection rate of the rapid BACpro II kit was improved when the viscosity of the ear discharge samples was eliminated. In 1-colony morphology, *C. diphtheriae* and *M. pachydermatis* were identified in two cases using 0.80 µm filtration, with bacterial counts of  $\leq 1.0 \times 10^4$  CFU/mL. In 2-colony morphology, using 0.80-µm membrane filtration with the rapid BACpro II kit, three samples were correctly identified: *B. cereus* and *S. schleiferi*, *M. pachydermatis* and *S. schleiferi*, and *P. aeruginosa* and *S. schleiferi*. Removing the sample's viscosity enabled its identification.

We found that 71.4% (25/35) of the isolated bacteria belonged to genus *Staphylococcus*, which was higher than in previous studies (Hariharan 2006; Lyskova 2007). *Staphylococcus* spp. are normal flora of the external ear canal, and thus, predisposing or primary factors are needed to cause otitis (Rosser 2004). *Malassezia pachydermatis* and *Staphylococcus pseudintermedius* were isolated from the same ear discharge sample. This is thought to occur due to a symbiotic relationship between these organisms. *Staphylococci* produce beneficial growth factors and change the environment to favor yeast growth (Miller 2012; Bajwa 2017). Microorganisms that are less frequently isolated from otitis ears include *Bacillus cereus*, *Corynebacterium diphtheriae*, *Pasteurella multocida*, and *Pseudomonas aeruginosa* (Lyskova 2007), and similarly, these microorganisms were found at rates of 8.6%, 2.9%, 2.9%, and 8.6% in the current study.

The conventional gold standard method for identifying bacteria in ear discharge cultures-quantitative culture on solid medium followed by biochemical characterization of isolates-requires 48 to 72 hours (Yang, 2018). In contrast, MALDI-TOF MS can identify bacteria from culture within 24 hours (Sogawa 2011). Additionally, bacterial species of ear discharge samples prepared using membrane filtration with the rapid BACpro II kit can be identified in approximately 30 minutes using MALDI-TOF MS. Therefore, this method may be a promising option for bacterial identification due to its speed.

In conclusion, MALDI-TOF MS with a membrane filter and rapid BACpro II kit is a speedy and reliable protocol for identifying bacteria in canine ear discharge samples in a clinical setting.

#### Author contributions

H. M., M. S., M. S. and K. S. performed all experiments. H. M., T. I., T. W. and K. S. analyzed the data and wrote the manuscript. H. M. and K. S. designed and directed the study and revised the manuscript. The final manuscript was reviewed and approved by all authors.

#### Conflict of interest

The authors declare no conflicts of interest.

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#### Ethical approval

The ethics committee for Maeda Veterinary Hospital approved the protocol (2023-01). All owners gave signed informed consent to participation of their animal in the study.

#### Data availability

Data are available on request from the corresponding author.

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