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Use of Magnetic Nanoparticles to Isolate Anaerobic Bacteria Anaerop Bakterilerin Tanımlanmasında Manyetik Nanopartiküllerin Kullanılması

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Özet:

Giriş: *Normal vücut florasının önemli bir bölümünü oluşturan anaerop bakteriler, yaşamı tehdit eden ciddi enfeksiyonlara yol açabilmektedir. Anaerop bakterilerin izolasyon ve identifikasyonları zaman alıcı ve zor yöntemler gerektirdiği için sadece belli klinik laboratuvarlarda yapılabilmektedir. Bu sebepten anaerop enfeksiyonların tanı ve tedavisi gecikmekte ve ampirik tedaviye bağlı ilaç direnci görülmektedir. Bu bakterilerin erken tanımlanmasını sağlayacak yeni yöntemler, tedavi süresinin ve anaerop enfeksiyonlarına bağlı ölüm oranlarının düşürülmesine yardımcı olacaktır.*

Method: *Bu çalışmada, anaerop bakterilerin hızlı tanımlanmasında kullanılabilecek yeni manyetik nanopartiküllerin tasarlanması amaçlanmıştır. Sentezlenen N - Metil - D - Glukamin (NMDG) bağlı manyetik nanoparçacıklar (Mag-NMDG), geçirimli elektron mikroskopisi (TEM), taramalı elektron mikroskobu (SEM), dinamik ışık saçılımı (DLS), titreşimli örnek manyetometrisi (VSM) ile karekterize edildi.*

Sonuç: *Sentezlenen Mag-NMDG nanopartikülleri, Actinomyces odontolyticus, Prevotella buccae, Veillonella parvula, Bifidobacterium dentium ve Bacteroides fragilis gibi kültür ortamından izole edilen gram pozitif ve gram negatif anaerop bakterilere uygulandı. Bakterilerin Mag-NMDG nanopartiküllere bağlanma durumu mikroskop görüntüleri, McFarland değerleri ve MALDI-TOF MS tanımlama skorları ve spektrumları ile tespit edildi.*

Tartışma: *Bu çalışma neticesinde, geliştirilen Mag-NMDG nanopartiküller anaerop bakterilerin numune ortamından direkt izole edilmesi ve tür düzeyinde tanımlanması için kullanılabileceği belirlendi. Böylece izolasyon ve tanımlama aşamalarındaki zaman alıcı ve zahmetli birçok basamak bertaraf edilebileceği öngörülmektedir.*

Anahtar kelimeler*: Manyetik nanopartiküller, Anaerop Bakteriler, Tanımlama, Bakteri yüzeyinin modifikasyonu*

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Abstract:

Introduction: *Anaerobic bacteria, which make up an important part of normal body flora, may lead to serious life-threatening infections. Since isolation and identification of anaerobic bacteria require time-consuming, sensitive and difficult methods, they can only be performed in certain clinical laboratories. For this reason, diagnosis and treatment of anaerobic infections are delayed and drug resistance is observed due to empirical treatment. New methods that will enable the early identification of these bacteria will help reduce the duration of treatment and mortality rates due to anaerobic infections.*

Method: *In this study, it is aimed to design magnetic nanoparticles attached to N-methyl-D-glucamine (Mag-NMDG) to catch anaerobic bacteria for rapid identification. Mag-NMDG nanoparticles were characterized by transmission electron microscopy (TEM), scanning electron microscopy (SEM), dynamic light scattering (DLS) and vibrating sample magnetometer (VSM).*

Results: *Mag-NMDG nanoparticles were applied to gram positive and gram negative anaerobic bacteria such as Actinomyces odontolyticus, Prevotella buccae, Veillonella parvula, Bifidobacterium dentium and Bacteroides fragilis isolated from culture media. The binding of bacteria to Mag-NMDG was determined by microscope images, McFarland values and MALDI-TOF MS identification scores.*

Conclusion: *As a result of this study, it was concluded that the Mag-NMDG nanoparticles could be used to isolate anaerobic bacteria directly from samples. Thus, it is foreseen that many time-consuming and troublesome steps in the isolation and identification stages can be eliminated.*

Keywords: *Magnetic Nanoparticles, Anaerobic Bacteria, İdentification, Bacteria Surface Modification*

Introduction

Anaerobic bacteria, the majority of which are members of the normal flora, are of great importance for human health. Anaerobic bacteria constitute the majority of the bacterial population in adult humans.[1] Mainly found genera are *Bacteroides, Lactobacillus, Fusobacterium, Bifidobacterium, Eubacterium, Peptococcus, Peptostreptococcus* and *Veillonella.*[2] Bacterial cell wall structures have been extensively studied because they are targets of antibiotics and interact with the human immune system.[3]

The cell walls of bacteria contain a wide variety of glycan structures, including teichoic acids (specific for gram positive organisms), lipopolysaccharides (specific for gram negative organisms), glycolipids, capsule polysaccharides and glycoproteins.[4] It is very difficult to isolate and identify anaerobic bacteria from clinical specimens in the laboratory. Anaerobic bacteria causing polymicrobial infections and accompanying secondary infections make it difficult to isolate and identify these bacteria from clinical samples. As a result, it makes the choice of antibiotics to be used against these bacteria seriously uncertain.[4] Accurate and rapid identification of anaerobic bacteria is important for the correct diagnosis, treatment and follow-up of the disease.

Today, many different methods are used to identify anaerobic bacteria. These methods are generally based on in vitro cultivation, morphological and biochemical analysis. However, these methods, which are used to obtain and identify bacteria purely, involve many sensitive and laborious steps that can last up to 3-4 days and are insufficient to identify certain species.[5,6] Besides, PCR, ribotyping, ELISA, microarray etc. molecular-based diagnostic methods are also available. Although the sensitivity and specificity of these methods are high, they have disadvantages such as high cost, trained personnel and intensive sample pre-treatment. These disadvantages of traditional and molecular-based methods can be overcome by using nanotechnological methods.[7,8] Nanotechnology is a low-cost technology that requires few samples and provides reliable results in a very short time.[9] Magnetic iron oxide nanoparticles produced with this technology have natural advantages such as ease of surface modification, precisely controllable sizes, and large surface areas.[9] Therefore, the use of nanotechnology in clinical diagnostic methods is gaining importance day by day. However, in order to use nanotechnological diagnostic methods as a real alternative to clinical diagnostic methods, various modifications should be made to these particles at the molecular level and the sensitivity and specificity of the method should be increased.[10,11]

The aim of this study is to rapidly isolate and identify anaerobic bacteria with N-Methyl-D-Glucamine (NMDG) attached magnetic nanoparticles (Mag-NMDG). For this purpose, magnetic nanoparticles were functionalized with NMDG and characterized by transmission electron microscopy (TEM), scanning electron microscopy (SEM), dynamic light scattering (DLS) and vibrating sample magnetometer (VSM). The Mag-NMDG nanoparticles could be used to isolate anaerobic bacteria directly from samples. Subsequently, Mag-NMDG nanoparticles were used in isolation and identification of anaerobic bacteria from samples.

Materials and Methods

1. Materials

Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), N-methyl-D-glucamine ($C_7H_{17}NO_5$), Ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), 3-bromopropyl trimethoxysilane ($C_6H_{15}BrO_3Si$), Toluene, anhydrous| ($C_6H_5CH_3$) and Ethanol (C_2H_5OH) were purchased from Sigma-Aldrich and all of them were of analytical grade. Different solutions at various concentrations used in different experiments were gained by dilution of the stock solution. Every reagent that used were of analytic grade and used as such.

2. Synthesis and Characterization of Mag-NMDG Nanoparticles

The Mag-NMDG nanoparticles was synthesized by a three-step procedure. In the first step, magnetic Fe_3O_4 nanoparticles were fabricated by co-precipitation method reported in literatüre.[12] In the second step, the surface of the magnetic Fe_3O_4 nanoparticles coated with a (3-Bomopropyl) trimethoxysilane by some modifications. Briefly, 1 g of magnetic Fe_3O_4 nanoparticles powder was dispersed in 25 mL of dry toluene by sonicator for 30 min. Then, 0,5 mL (3-Bomopropyl) trimethoxysilane was added and the solution was refluxed at 60 °C and for 18 h.

Fe_3O_4@SiO_2-Br was washed with toluene several times, then separated by using neodymium magnet, final product dried by vacuum freeze dryer.[13,14] In final step, the obtained Fe_3O_4@SiO_2-Br (1 g) as supported was dispersed in 10 mL of water, and then NMDG (0.780 g) was added to the reaction mixture and this mixtrure was refluxed for 24 h. finally Mag-NMDG separated by magnetic decantaion and washed with deionized water to remove the unreacted chemicals and substances and then dried on a freeze dryer.[15,16]. Reaction sequences of synthesis for Mag-NMDG are shown in Figure1.

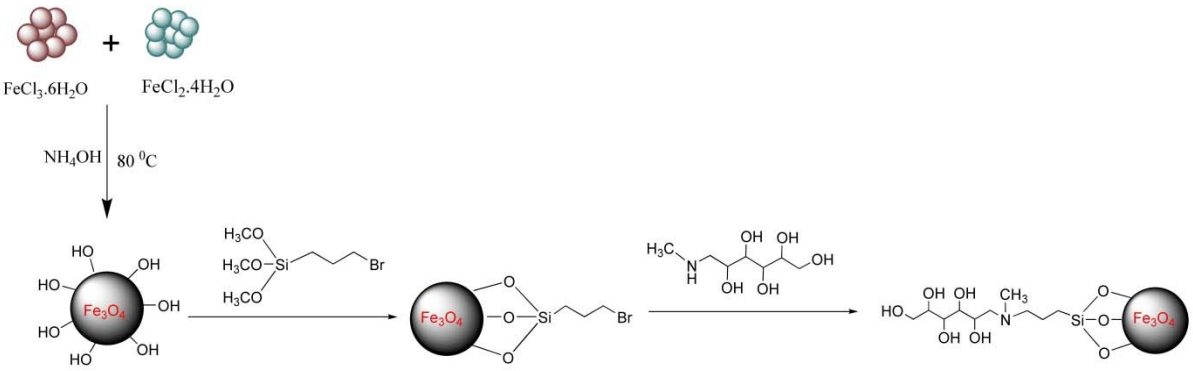

Figure 1. *The synthesis mechanism of Mag-NMDG nanoparticles*

3. Sample Cultivation, Bacteria Isolation and Identification

Samples requested for anaerobic culture from various clinics were sent to the bacteriology laboratory via sterile glass tubes, sterile plastic containers or sterile syringes. All clinical samples were inoculated onto anaerobic blood agar (BD *Brucella* Agar 5% Sheep Blood, 1 mg/L vitamin K1, 5 mg/L hemin, Becton Dickinson, Franklin Lakes, NY) and thioglycolate broth (BD Fluid Thioglycolate Medium, Becton Dickinson), incubating all media at 35-37 °C for 5 days.

Gram staining of all isolated anaerobic bacteria species was performed and images were taken under a light microscope. In addition, identification of all isolates was carried out using MALDI-TOF MS (Bruker Biotyper, Bellerica, MA, USA). Identified bacterial species are: *Bacteroides fragilis, Prevotella buccae, Veillonella parvula, Bifidobacterium dentium, Actinomyces odontolyticus.*

4. Detection of Anaerobic Bacteria Using Mag-NMDG Nanoparticles

McFarland, Gram stain and MALDI-TOF MS methods were used for the determination of bacterial isolation with Mag-NMDG. The isolation and detection stages of anaerobic bacteria with Mag-NMDG nanoparticle are shown in Figure 2.

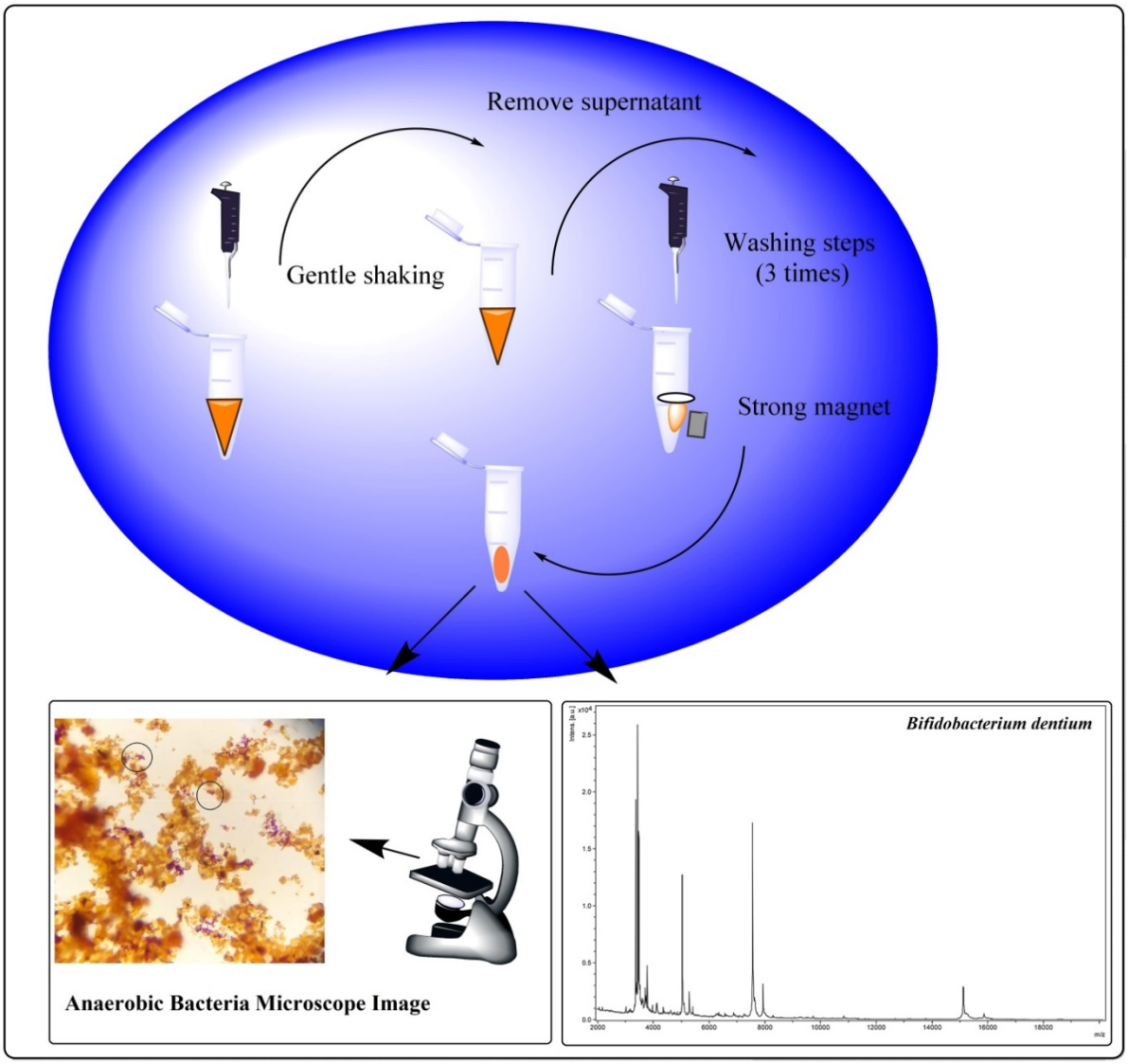

Figure 2. *Isolation and Detection Stages of Anaerobic Bacteria Using Mag-NMDG Nanoparticle*

5. Ethical Procedures

The Project titled as " Antibiotic susceptibility patterns of gram-negative anaerobic bacteria isolated from clinical specimen" planned by Selahattin ATMACA, Alican BILDEN, Nida OZCAN has been approved by the Ethics Committee of Dicle University Faculty of Medicine.

Result

1. Characterization of Mag-NMDG Nanoparticles

The magnetic properties of Mag-NMDG nanoparticle was researched by VSM analysis at room temperature. The saturation magnetization of Mag-NMDG was measured as approximately 38.2 emu/g (Figure 2A). The saturation magnetization value of Mag-NMDG is suitable for magnetic separation. Agglomerate size states of the synthesized Mag-NMDG nanoparticles were analyzed by DLS by providing dispersions in water. For the reliability of the results, each sample was distributed in the sonicator prior to the analysis was performed. Figure 2B shows the agglomeration size of Fe_3O_4, Fe_3O_4@SiO_2-Br and Mag-NMDG by DLS. As shown in Figure 2B, the particle sizes of Fe3O4, Fe_3O_4@SiO_2-Br and Mag-NMDG nanoparticles were measured to be approximately 141, 479 and 724 nm respectively.

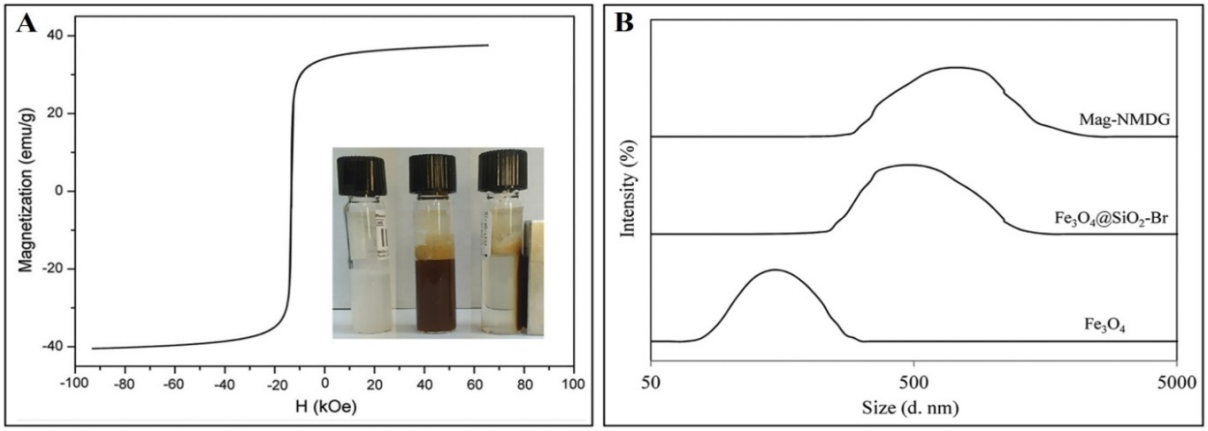

Figure 3. *(A) Magnetization curve of Mag-NMDG against magnetic field, (B) Size distribution graph of Fe_3O_4, Fe_3O_4@SiO_2-Br and Mag-NMDG nanoparticles*

TEM image of Mag-NMDG is given in Figure 3A. The size and shape analysis of the synthesized Mag-NMDG nanoparticles were analyzed with the TEM device. The nanoparticle size of Mag-NMDG was determined to vary between 12-16 nm.

SEM image of Mag-NMDG is given in Figure 3B. This image contains similar spherical images confirming that the surface morphology of Mag-NMDG nanoparticles is similar. These findings are consistent with DLS analysis results.

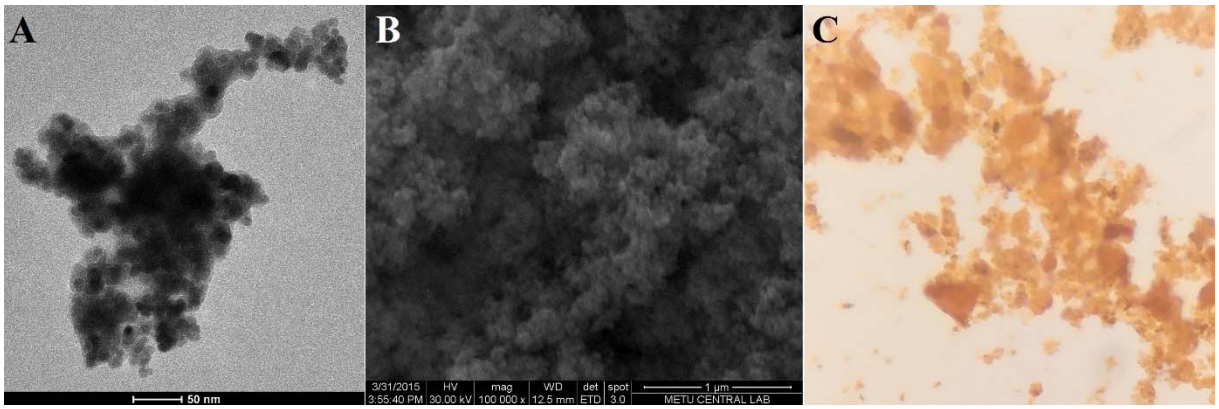

Figure 4. *(A) TEM image and (B) SEM image C)* **Optical microscopy images** *of Mag-NMDG nanoparticles*

2. Detection by McFarland Method

The McFarland turbidity standard value of bacteria grown in culture was set to 1.0 (3×10^8 cfu/ mL) and the results were given (Table 1). 4 mL of liquid was taken from the samples whose McFarland turbidity values were adjusted as 1.0 (3×10^8 cfu/mL) and transferred to tubes containing 10 mg Mag-NMDG. In order to occur the Mag-NMDG-bacteria complex, the tubes were vortexed for 5 minutes and kept at room temperature for 30 minutes. After the time, the Mag-NMDG-Bacteria complex was precipitated with the aid of a magnet. After magnetic separation was completed, the supernatant was transferred to another tube, McFarland values were determined, and the results were given in Table 1.

3. Detection by MALDI-TOF MS

The MALDI-TOF MS method was used to investigate whether the Mag-NMDG method could be used for species identification. First, the identification of anaerobic bacteria grown in culture was done with MALDI-TOF MS (Table 1). The same bacteria were then treated with Mag-NMDG nanoparticles. The bacterial species in the bacteria-Mag-NMDG complex taken from the pellet formed after the magnetic separation process were again identified by MALDI-TOF MS and the obtained scores were given (Table 1). Mass spectra obtained after identification with MALDI-TOF MS are shown in Figure 5. In our study, it was observed that the MALDI-TOF MS identification scores obtained before and after the experiment as a result of the treatment of isolated bacteria with Mag-NMDG nanoparticles were very close to each other. This shows that Mag-NMDG nanoparticles can be used together with the MALDI-TOF MS method for the identification of anaerobic bacteria.

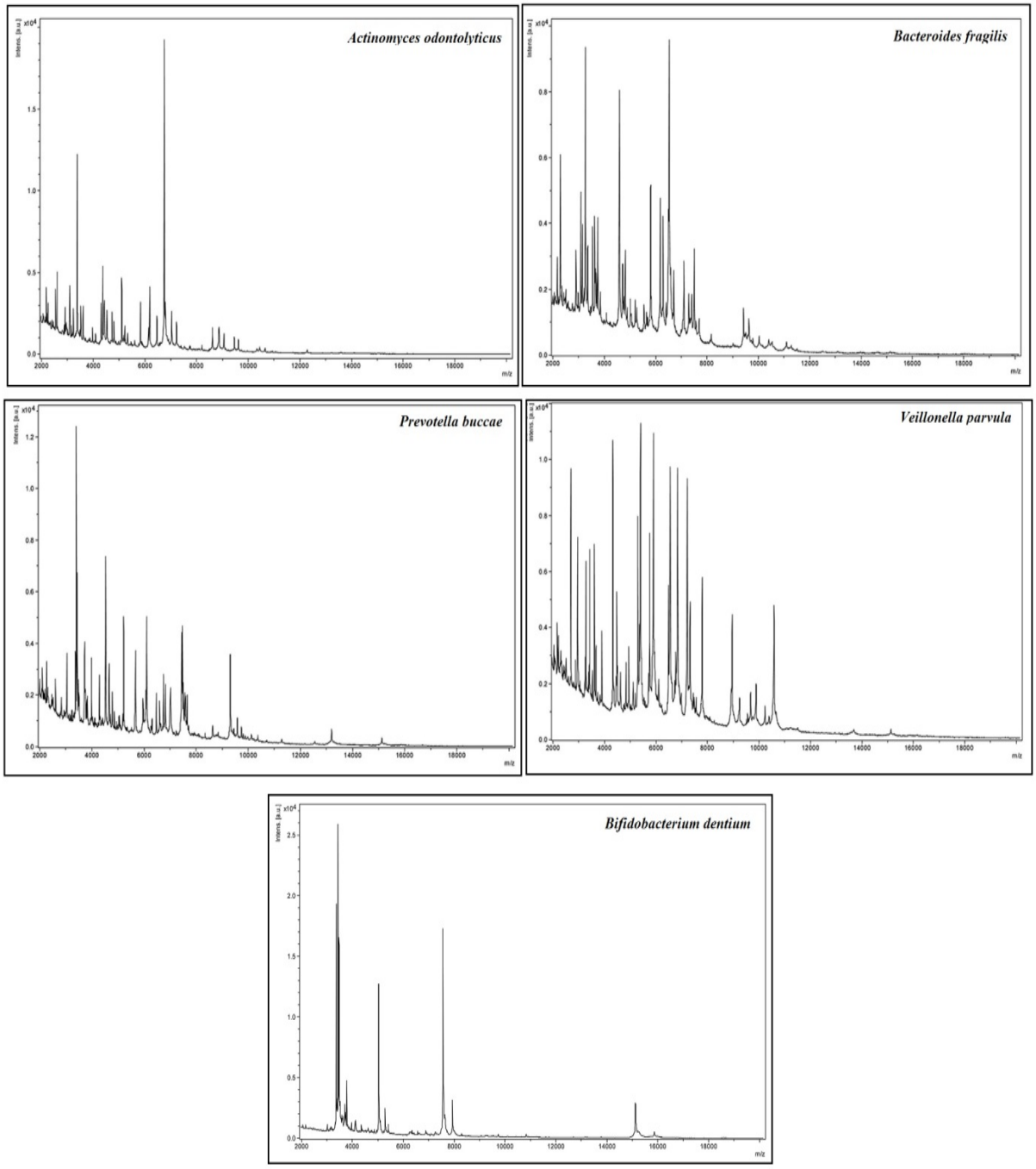

Figure 5*. Mass spectra of the Bacteria-Mag-NMDG complex obtained by MALDI-TOF MS*

4. Detection by Gram Stain

Gram staining was performed from the pellet to view the bacteria-Mag-NMDG complex formed after the magnet separation, and the formation of the bacteria-Mag-NMDG complex was observed under the light microscope (Figure 6, Figure 7, Figure 8, Figure 9, Figure 10).

Table 1: *Mcfarland and MALDI TOF MS Results of Anaerobic Bacteria Before and After the Experiment*

Anaerobic Bacteria	MALDI TOF MS		McFarland	
	Before Experiment	After Experiment Pellet	Before Experiment	After Experiment Supernatant
Bacteroides fragilis	2.304	1.831	1.08	0.81
Prevotella buccae	2.017	1.905	0.98	0.61
Veillonella parvula	2.320	1.827	1.04	0.64
Bifidobacterium dentium	1.901	1.841	1.01	0.53
Actinomyces odontolyticus	2.050	1.990	1.04	0.46

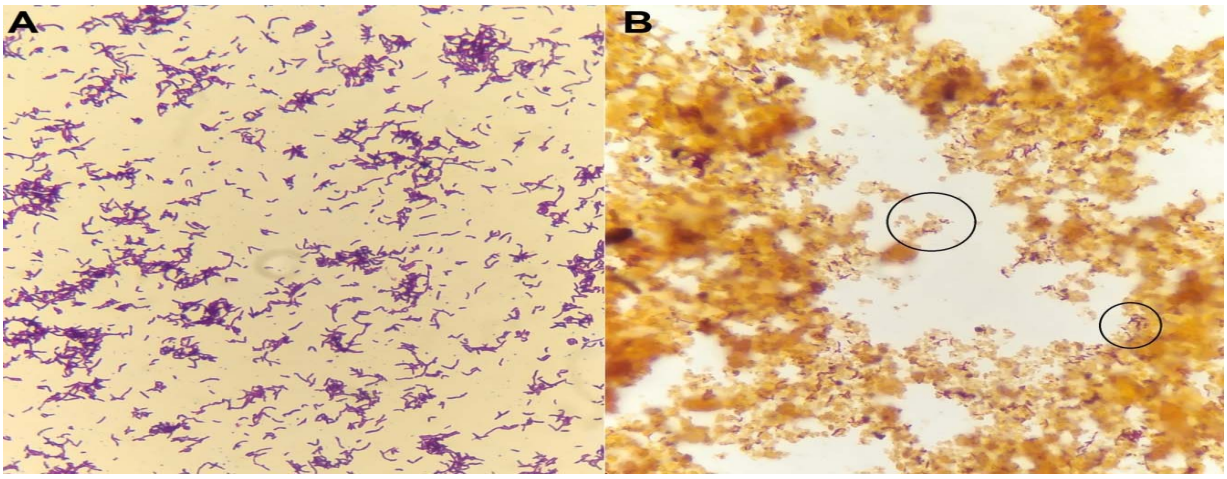

Figure 6. *(A) Actinomyces odontolyticus, (B) Actinomyces odontolyticus- Mag-NMDG*

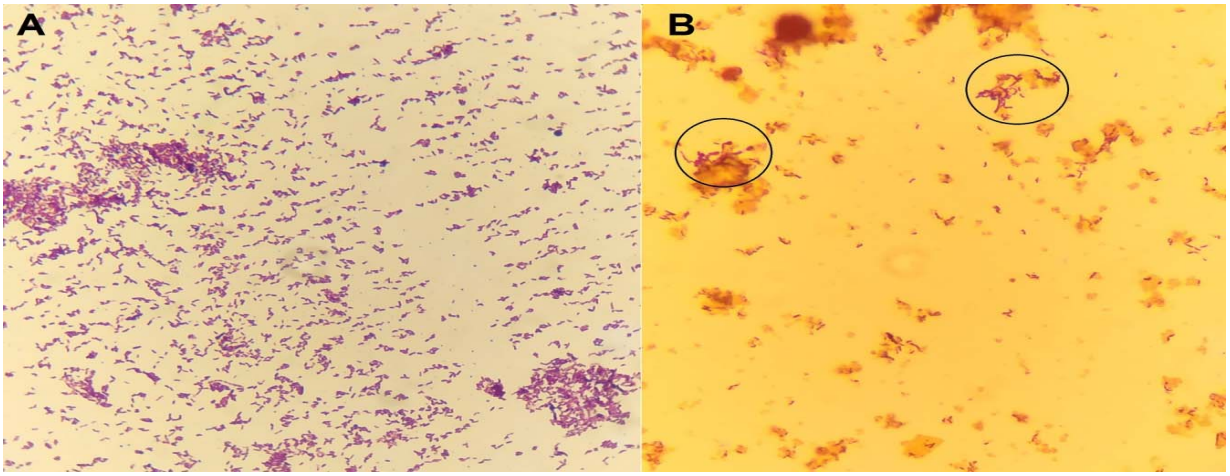

Figure 7. *(A) Bifidobacterium dentium, (B) Bifidobacterium dentium- Mag-NMDG*

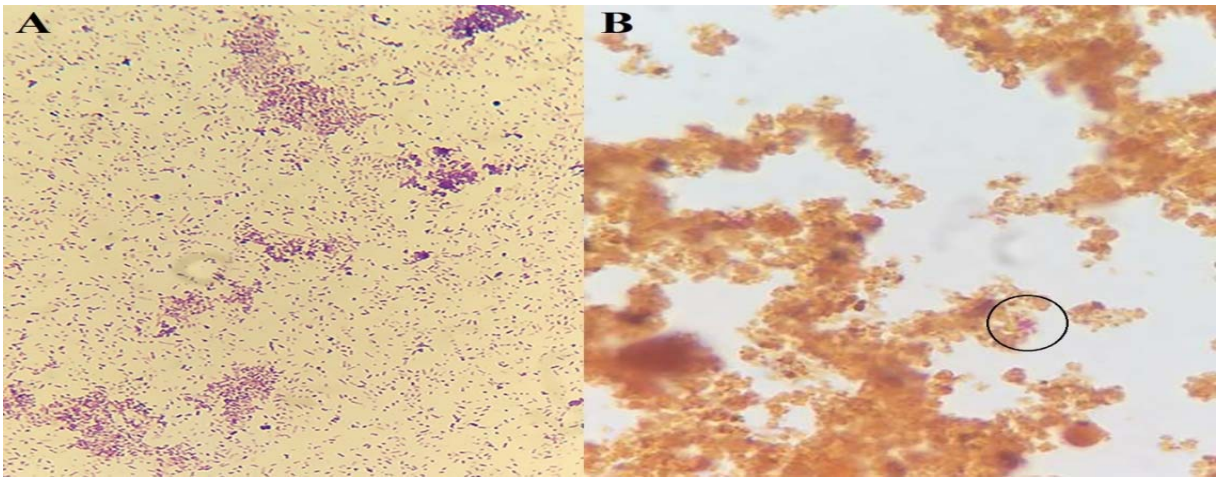

Figure 8. *(A) Prevotella buccae, (B) Prevotella buccae - Mag-NMDG*

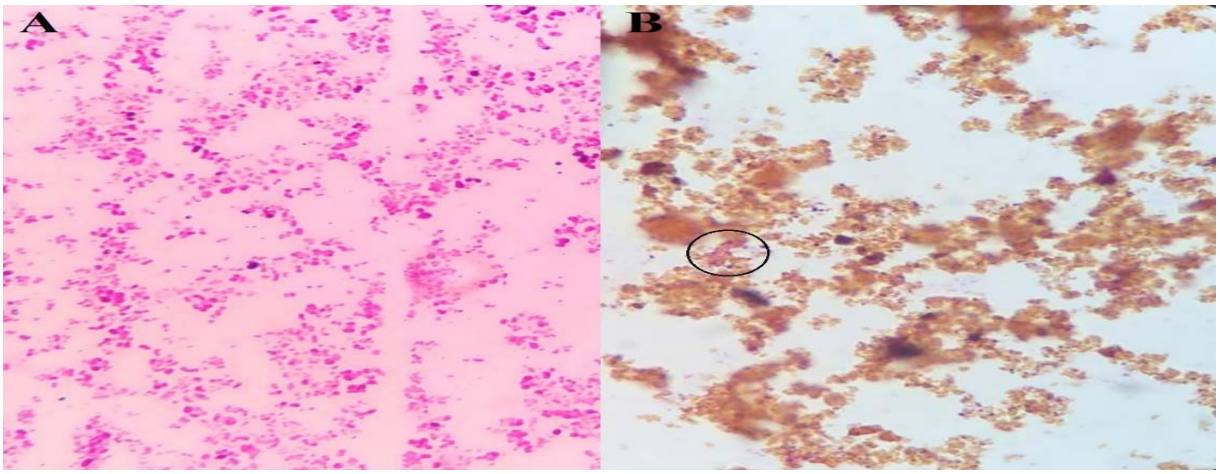

Figure 9. *(A) Veillonella parvula, (B) Veillonella parvula - Mag-NMDG*

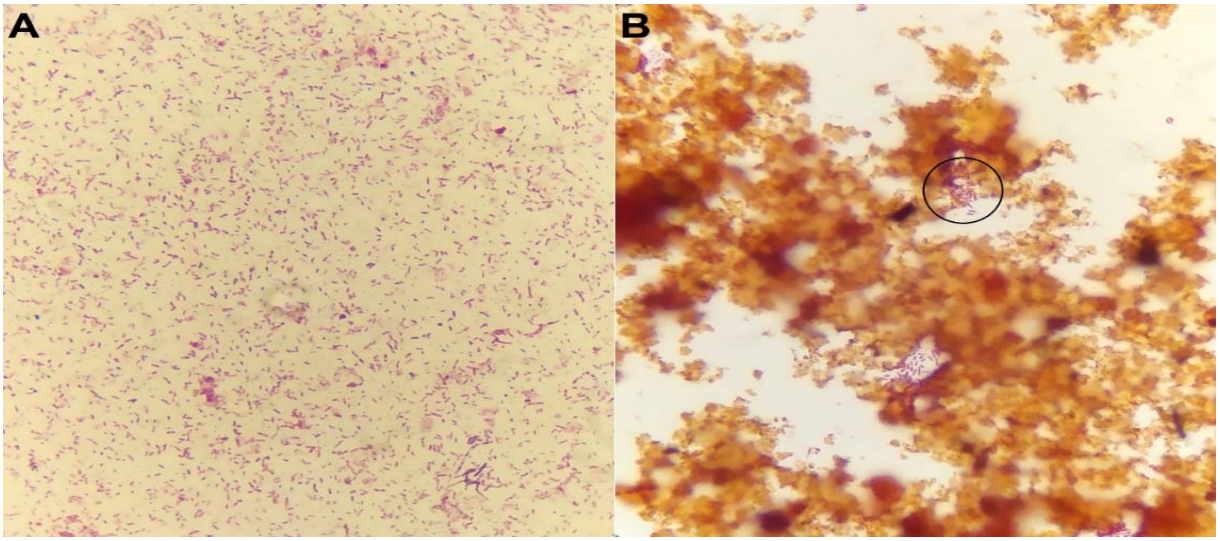

Figure 10. *(A) Bacteroides fragilis, (B) Bacteroides fragilis - Mag-NMDG*

Conclusion

Magnetic nanoparticles have the ability to bind to biological structures. Therefore, it has great potential in the detection of bacteria. Magnetic nanoparticles formed in appropriate proportions provide the opportunity to catch bacteria and isolate them from the environment. This approach has provided an attractive and innovative method for bacterial diagnostic methods.[5,6] The cell walls of bacteria contain a wide variety of glycan structures, including teichoic acids (specific to gram-positive organisms), lipopolysaccharides (specific to gram-negative organisms), glycolipids, capsule polysaccharides, and glycoproteins. These structures contain some functional groups such as (–COO−), (–NH) , (–OH), (–C=O), (–C–N–), (–C–O), (–C–H). Each of these groups has different affinity and adsorbs different molecules.[13] Studies on the use of nanoparticles in the detection of bacteria are increasing day by day. Suaifan et al[17]. measured the color change based on proteolytic enzyme activity of *Staphylococcus aureus* using magnetic nano beads and thus made quantitative analysis of the bacteria in the sample. In the study of Tural et al[13]., they successfully prepared a new magnetic biosorbent by immobilizing *Bacillus subtilis* with nano-sized magnetic silica. Thus, they used this biosorbent to remove methylene blue pollution. Gautam et al[3]. showed in detail how bacterial cell surface structures can be modified with functional groups on different bacterial species in their study. All these studies show that bacteria-specific components such as enzymes and cell wall structures can make specific bonds with nanoparticles, and this complex can be used in many different areas, including bacterial identification.

Therefore, the formation of the bacteria - Mag-NMDG complex appears to depend on the affinity of these functional groups. In our study, it was observed that magnetic nanoparticles functionalized with NMDG can bind to the cell wall structures of Gram-negative and Gram-positive anaerobic bacteria and can thus be isolated from samples. As a result, as seen in Table 1 in our study, the McFarland and MALDI TOF MS values of Gram-negative and Gram-positive anaerobic bacteria isolated by traditional methods before and after the experiment also show that the functional groups can combine with these bacterial groups under appropriate conditions.

According to McFarland results, Mag-NMDG nanoparticles provided high adhesion to all bacterial species in our study. The pellet was transferred to the MALDI TOF MS device for species identification. MALDI TOF MS identification scores were determined at the species level. In addition, the formation of the bacteria-Mag-NMDG complex is shown by the optical microscope images given in Figures 5, 6, 7, 8 and 9. These results obtained in our study show that the nanoparticles we have developed can be used for the isolation and identification of certain anaerobic bacteria from the samples.

As a result; we succeeded in attaching the Mag-NMDG nanoparticles we had developed to anaerobic bacteria such as *Bacteroides fragilis, Prevotella buccae, Veillonella parvula, Bifidobacterium dentium, Actinomyces odontolyticus.*

We demonstrated that Mag-NMDG nanoparticles can be used in a collaborative manner with MALDI-TOF MS to identify anaerobic bacteria at the species level. Since our research is a proof of concept for Mag-NMDG, specificity / cross-reactivity experiments have not been conducted, but these will be part of future studies. With this study we have done, nanotechnology; we believe that developing powerful combinations with existing techniques for the early and rapid detection of bacteria in samples will become more sensitive and cost-effective than current laboratory techniques.

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