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RESEARCH ARTICLE

Investigation into the Usability of the *Maackia amurensis* Lectin in Bacterial Diagnosis with the Help of Transmission Electron Microscope

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ABSTRACT

Objective: Glycoconjugates are known to play a crucial role in the attachment-recognition relationship of prokaryotic cells, particularly bacteria. Therefore, understanding the function and properties of these glycoconjugates is of great importance.

Materials and Methods: While various methods have provided significant information in determining the chemical structures and functions of sialic acids, histological methods have remained essential in determining their function and location in living organisms. The discovery of lectins with unique marking ability based on the bond structures of sialic acids and the development of antibodies, which can be microscopically distinguished by adding colloidal silver and gold particles, were significant milestones. In the 1990s, methods were developed to specifically mark the types and locations of sialic acids using immunocytological, histological, and fluorescence microscopy methods with fluorescently labeled lectins and antibodies. These methods still remain valid and important today. Using this information, it is possible to create smart drugs and biomarkers specific to bacteria.

Results: In this study, the unique connections of gold-labeled lectins with different bacteria were demonstrated with the help of transmission electron microscopy.

Conclusion: Our study supports the idea that labeled lectins could be used for rapid and precise bacterial diagnosis.

Keywords: Sialic acid, Bacteria, Biomarkers, Immunocytochemistry, Microscopy

INTRODUCTION

"Glycans", often referred to as oligosaccharides and polysaccharides, are found in all eukaryotic and prokaryotic cells, as well as viruses, including embryonic and pluripotent stem cells. They consist of a basic chain structure with added protein and lipids. When found on cell surfaces, glycans form the "Glycocalyx" layer.[1–5] This layer plays a crucial role in various cellular processes such as cell growth, differentiation, cell migration, disease pathogenesis, and immune system function.[1–3,5] One of the most significant glycans are sialic acids, which were initially discovered in saliva during the mid-1930s and subsequently referred to as sialic acid. In 1941, sialic acid was also identified in nerve cells and termed neuraminic acid. It was later established in the 1950s that sialic acid and neuraminic acid were identical structures consisting of 9-carbon acidic sugar molecules with a pH range of 2-3.[1] N-linked and O-linked glycans, as well as glycosphingolipids, are commonly situated at the outermost ends of biological systems.[1,5,6] When considering the tasks that are solely attributed to their receptor functions, several come to mind. These include primarily the formation of viral and bacterial infections, toxin binding, colony formation of bacteria, determination of serological characteristics, and the preservation of cell shape.[1,5,6]

One of the most commonly utilized methods for labeling surface glycoconjugates and unique sugar sequences is through the use of lectins. Lectins were first discovered in castor bean plant seeds in 1888. These molecules, which have the ability to agglutinate animal red blood cells, have attracted attention with this feature. Although its specific glycan binding properties are known, its biological functions in plants and other organisms are mostly unknown. Different classifications of lectins were later made, such as R-Type, L-Type, P-Type, C-Type, I-Type, Galectins and Bacterial Lectins. The *Maackia amurensis* lectin used in our study is an L-Type lectin and specifically binds to sialylated glycans.[5–7]

Lectins that are utilized to label sialic acids can be obtained from a variety of sources including vertebrates, arthropods, mollusks, protozoa, plants, bacteria, and viruses.[5] Lectins have been found to be useful in the detection of glycoconjugates in complex matrices and body fluids, even in trace amounts.

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Additionally, they can be utilized to characterize compounds that bear a specific glycan structure. The mitogenic stimulation of lymphocytes and lectins in cell division can be leveraged due to their distinct and specific binding ability to detect glycostructures, virus and bacteria surfaces, and changes in cancer cells after malignant transformation. These changes may occur in cellular or non-cellular membrane elements or within the cell itself.[8–10]

The *Maackia amurensis* plant lectin (α2-3) utilized in our study is a lectin that specifically recognizes the motif [Siaα2-3Galβ1-4GlcNAc] from bound sialic acids. The *Maackia amurensis* lectin [MAL] demonstrates a unique ability to oligovalently bind (α2-3) sialic acids with high affinity. Conversely, it exhibits a weaker binding affinity towards sialic acids with monovalent (α2-3) linkages.[11]

The identification of bacteria at both the genus and species level was initially performed using morphological characteristics, followed by biochemical tests. However, these traditional methods are time-consuming, taking hours and days to complete. Therefore, molecular biological methods have gained importance in bacterial classification as a result of technological advancements. These methods are based on determining genes and their products and can be categorized into seven main groups. These include DNA-based methods such as 23S, 16S, and 5S rRNA sequence similarity, cataloging of oligonucleotides, analyses for the separation of total soluble proteins that form morphological and biochemical properties, cell wall analysis, serological profiles, and profiles of cellular fatty acids.[7,12,13]

The combined use of traditional and molecular biology-based techniques has facilitated the rapid classification of bacteria and the identification of new microorganism groups. In addition to these methods, automated classification devices such as the VITEK 2 Compact (bioMérieux Diagnostics) microbial identification and API identification systems are commonly used.[12] The VITEK 2 Compact system utilizes colorimetric cards containing 60-65 different tests to determine microorganisms at the species level within 5-8 hours.[13]

In our study, we examined bacterial surface differences using labeled lectins that could make highly specific connections. The feasibility of glycobiology-based bacterial identification was evaluated based on these bacterial cell surface differences. The originality of our work; the aim of this study is to examine the possibility of rapid and accurate bacterial species diagnosis by using the sensitive binding abilities of lectins to glycoconjugates. The method sensitivity was demonstrated by transmission electron microscopy. Based on the results of our study, we believe that the methods developed using labeled lectins can provide more sensitive results than the VITEK and API systems in bacterial species identification.

MATERIALS AND METHODS

In our study, we employed the *Maackia amurensis* lectin, a lectin that selectively recognizes the motif [Siaα2-3Galβ1-4GlcNAc] present on sialic acids (α2-3), as a marker to label bacterial samples from five different strains with three distinct cell morphologies and genera. To achieve this, we conjugated the *Maackia amurensis* lectin with colloidal gold particles measuring 10-12 nm, which were synthesized in our laboratory. The [Siaα2-3Galβ1-4GlcNAc] motif was specifically labeled on the cell membranes of bacteria using the colloidal gold-linked lectin.

To prepare the bacterial samples for microscopy, they were first fixed and embedded in epoxy resin. Thin sections were then obtained using a Reichert OM U3 ultramicrotome and micrographed using a JEOL 1010 transmission electron microscope.

The study employed five bacterial strains of three different cell morphologies and genera, including Gram-negative, coccobacillary strains of *Escherichia coli* ATCC 35860 (K92), ATCC 8739, and ATCC 29998, *Micrococcus luteus* ATCC 9341 in coccal form, and *Bacillus subtilis* ATCC 6634 bacteria in bacillus form.

The *Escherichia coli* ATCC 35860 (K92) strain (BOS12 strain) was isolated from cerebrospinal fluid and is used in sialic acid production and bacteriophage host assays. The *Escherichia coli* ATCC 8739 strain was isolated from feces and is utilized in various microbial tests, including media tests, impact tests, and quality tests. The *Escherichia coli* ATCC 29998 strain is a clinical isolate, particularly used in spectinomycin action spectacin tests and nutritional analysis. The *Micrococcus luteus* ATCC 9341 strain, currently known as the *Kocuria rhizophila* ATCC 9341 strain, is a soil isolate and is frequently used in antibiotic tests. The *Bacillus subtilis subsp. spizizenii* ATCC 6633 strain is a clinical isolate and is also utilized in antibiotic tests.

Bacteria were grown using Luria Broth (LB) and Luria Agar (LA) media. Broth inoculated with a single colony of bacteria grown for appropriate times on solid media was grown at 37 °C until sufficient optical density was achieved and stored in 50% glycerol at -20 °C and 15% glycerol at -80 °C until the marking step.

Preparation of Colloidal Gold

The preparation of colloidal gold followed the procedure outlined in Hayat.[14] To form gold-labeled lectins, a stock colloidal gold solution was first prepared using tetrachlorogold (III). Then, 5 mL of Na-citrate was added to boiling water, followed by the appropriate amount of 0.2% tetrachloroauric acid (gold). The mixture was allowed to boil for 5-10 min, during which red-colored colloidal gold particles were formed. The forma-

tion of the particles was confirmed by spectroscopic scanning ($OD_{400-600}$).

To control the agglutination of colloidal gold, 1% PEG 20,000 and 100 mM K_2CO_3 were added to 5 mL of the gold solution, and the pH of the solution was measured. The amount required for agglutination was determined, and the prepared colloidal gold was then challenged with the *Maackia amurensis* lectin.

Gold-Lectin Conjugation

The gold solution was prepared by adding 100 mM K2CO3 to particle-free glassware on a magnetic stirrer. Then, lectin was added dropwise to the mixture, followed by 1% PEG 20,000. The resulting mixture was centrifuged at 14,000 rpm at 4 °C, and the supernatant was discarded. The precipitates were then diluted with a mixture of PBS and PEG. The conjugates were scanned spectrophotometrically to determine the optimal amount for use.

Exposing the Bacteria to Colloidal Gold-Labeled Lectins

Bacteria were cultured in LB medium at the appropriate temperature. Samples (500 μl) were taken from each bacterial culture and transferred to sterile Eppendorf tubes. The samples were centrifuged at 7,000 rpm for 10 min, and the supernatant was discarded. The obtained precipitates were resuspended in 500 μl of salt solution (PBS pH 7.2/PEG 20,000). A 15 μl part of the samples was separated from the cells, centrifuged at 7,000 rpm for 10 min, and washed by adding 500 μl of salt solution (PBS/PEG 20,000) in sterile Eppendorf tubes.

Salt solution (10 μl) and gold-labeled lectins (10 μl) were added to each tube, and the appropriate storage conditions were observed. The samples were then fixed using TEM fixation steps and embedded in Epoxy resin.[7] Thin sections with a thickness of 250-300 Å were taken using the Reichert OM U3 ultramicrotome and stained with saturated uranyl acetate and 2.66% lead citrate. The samples were examined at 80 kV in a JEOL 1010 electron microscope, and micrographs were taken and evaluated for results.

RESULTS

In this study, we utilized colloidal gold-conjugated *Maackia amurensis* lectin (MAL) to specifically detect the motif [Siaα2-3Galβ1-4GlcNAc] (α2-3). MAL is known to especially bind oligovalently-bound (α2-3) sialic acids.11 It uniquely marked the [Siaα2-3Galβ1-4GlcNAc] motif observed on the bacterial surfaces following the washing steps (Figures 1-5). When the results of the bacteria used in our study are examined, we see the presence of sialic acids with the [Siaα2-3Galβ1 -4GlcNAc] motif in the cell membranes of the coccobacillus *Escherichia coli* ATCC 35860 (K92) strain (Figure 1), *Escherichia coli* ATCC 8739 strain (Figure 2), and *Escherichia coli* ATCC 29998 strain (Figure 3). It should be noted here that all the bacteria samples examined in this study have the [Siaα2-3Galβ1-4GlcNAc] motif in their membranes, but the densities of oligovalently-bound (α2-3) sialic acids were different even among the various strains of the same bacteria. The *Escherichia coli* ATCC 35860 (K92) strain (Figure 1) and *Escherichia coli* ATCC 8739 strain (Figure 2) specifically exhibited more intense labeling indicating a higher concentration of the [Siaα2-3Galβ1-4GlcNAc] motif. On the other hand, oligovalently-bound (α2-3) sialic acids were found to be quite low in the *Escherichia coli* ATCC 29998 strain (Figure 3).

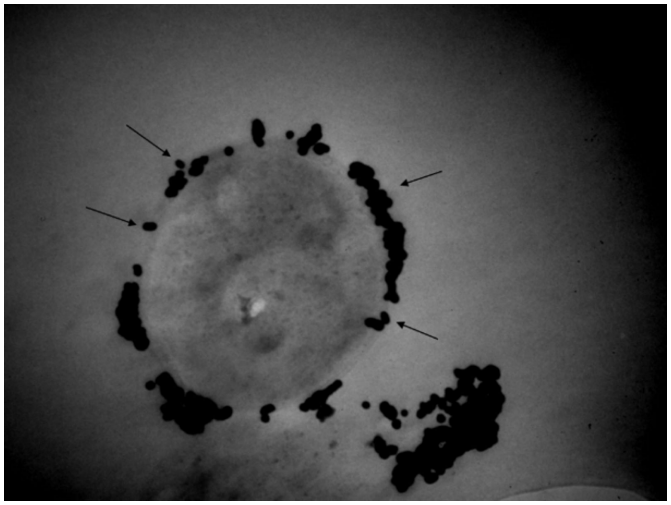

Figure 1. The *Escherichia coli* ATCC 35860 (K92) strain, exhibiting a coccobacillus structure (α2-3) with sialic acids, which were marked with gold-labeled MAL (→) at a magnification of 100,000X.

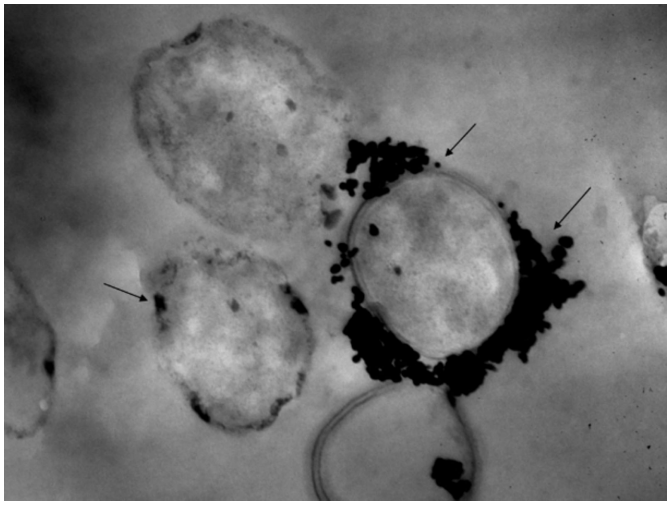

Figure 2. The *Escherichia coli* ATCC 8739 strain, exhibiting a coccobacillus structure (α2-3) with sialic acids, was visualized using gold-labeled MAL (→) at a magnification of 75,000X.

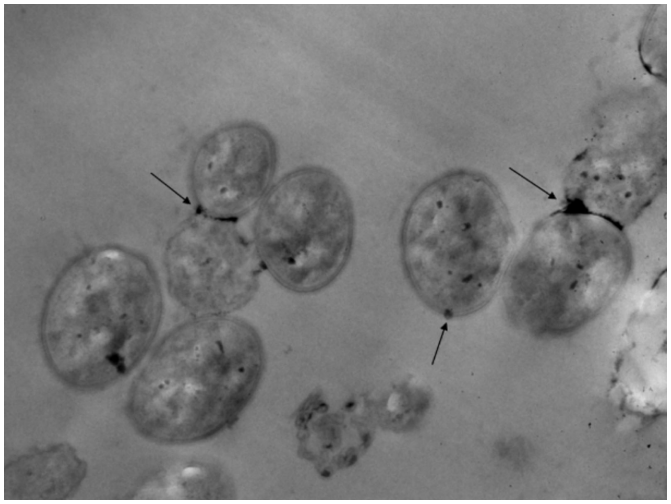

Figure 3. The *Escherichia coli* ATCC 29998 strain, exhibiting a coccobacillus structure (α2-3) with sialic acids, was visualized using gold-labeled MAL (→) at a magnification of 30,000X.

The presence of sialic acid bearing the motif [Siaα2-3Galβ1-4GlcNAc] in the coccus form of *Kocuria rhizophila* ATCC 9341 (formerly known as *Micrococcus luteus* ATCC 9341) is observed to be in a very limited quantity, as depicted in Figure 4.

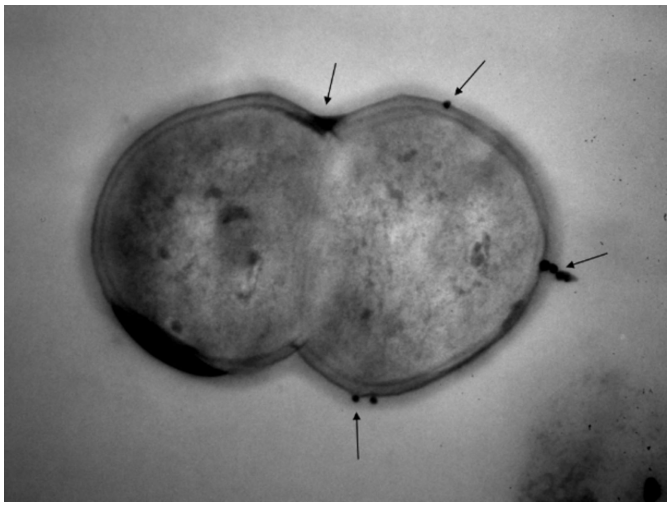

Figure 4. The coccus form of *Micrococcus luteus* ATCC 9341, now classified as the *Kocuria rhizophila* ATCC 9341 strain, displays α2-3 sialic acids labeled with gold using MAL (→) at a magnification of 100,000X.

In the study, it was observed that *Bacillus subtilis* ATCC 6633 cell membrane, in the form of bacillus, contained oligovalently-bound (α2-3) sialic acids which were highly labeled with colloidal gold bound - MAL (Figure 5).

DISCUSSION

The *Maackia amurensis* plant lectin used in our study is a lectin that specifically recognizes the oligovalently-bound

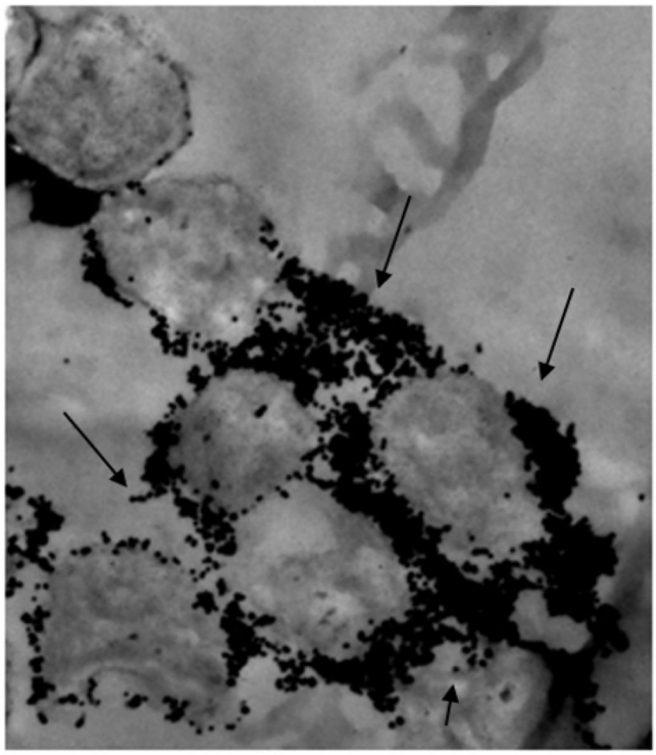

Figure 5. The presence of sialic acids in *Bacillus subtilis* ATCC 6633 strain (α2-3) in its bacillus form was confirmed through gold labeling with MAL (→) at a magnification of 30,000X.

(α2-3) sialic acid motif [Sia2-3Galβ1-4GlcNAc]. The lectin was conjugated with colloidal gold, which was prepared in our laboratory. We investigated bacteria with various cell morphologies, including coccus, coccobacillus, and bacillus forms. The bacterial strains used in this study were Gram-negative coccobacillus *Escherichia coli* strains ATCC 35860 (K92), ATCC 8739, and ATCC 29998, and Gram-positive coccus form *Micrococcus luteus* ATCC 9341, and bacillus form *Bacillus subtilis* ATCC 6634. These morphologies are the most common in bacteria, some of which may have pathogenic properties. These are important species that are frequently encountered in microbiological studies. The lectin-labeling of bacteria was visualized ultrastructurally (Figures 1-5) and quantified to determine the amount of oligovalently-bound (α2-3) sialic acids in different bacterial genera and morphologies (Figures 1, 4 and 5). Specifically, we compared the amount of [Siaα2-3Galβ1-4GlcNAc] sialic acid motif in *Escherichia coli*, *Micrococcus luteus* ATCC 9341, and *Bacillus subtilis* ATCC 6634 strains, as well as in different species of Gram-negative coccobacilli belonging to the same genus (ATCC 35860 [K92], ATCC 8739, and ATCC 29998) (Figures 1-3) of Gram-negative, coccobacillary *Escherichia coli* of different species belonging to the same genus (Figures 1-3). Our results provide serologically sensitive information about the genus and species of bacteria based on the specific labeling of surface glycoconjugates with lectins. The results were verified visu-

ally and ultrastructurally by micrographs (Figures 1-5), which supported previous findings in the literature.[15–17]

Studies on sialic acid and its metabolism in prokaryotes have mainly focused on gene regions associated with capsule synthesis due to their involvement in disease-causing properties.[16–19] Although different bacteria (e.g., *E. coli* K1, K12, K92, O24, O37, O104, O56, O157, O111, BOS12, *Neisseria meningitidis, Haemophilus influenzae, Campylobacter jejuni, Staphylococcus epidermidis, Streptococcus pneumonia, Brucella, etc.*) exhibit striking similarities in determinant organization; the degree of virulence is determined by the existence and type of polysaccharides on the surface of cell membrane.[1,5,7] Based on the findings of our study, we have obtained supportive evidence regarding pathogenicity at the ultrastructural level. Our results indicate that the surface of the selected bacteria contained a higher concentration of sialic acid with an increase in the range of bacteria used. Notably, the *Bacillus subtilis* ATCC 6634 strain demonstrated the most significant labeling, followed by the *Escherichia coli* strains ATCC 35860 (K92), ATCC 8739, and ATCC 29998, which exhibited similar but less pronounced labeling (Figures 1-3 and 5).

This study demonstrates the feasibility of distinguishing between different bacterial species using *Maackia amurensis* lectin, which selectively binds to α2-3 sialic acid units on bacterial surfaces. The observed variations in sialic acid density among strains of the same *Escherichia coli* species suggest that this method can also differentiate between strains within a species. The visual results obtained provide evidence that lectins can provide specific and precise binding, enabling accurate distinction between genera and species. These findings are consistent with previous studies in the literature.[1,5]

CONCLUSION

Transmission electron microscopy has demonstrated that bacterial diagnosis can be achieved at the genus and species level by utilizing specific lectins. This method is based on glycobiology and employs labeled lectins to form unique connections with sialic acid units. These findings suggest that immunofluorescent labeled lectins could be used to develop a practical, rapid, and specific bacterial diagnosis method.

Based on these findings, we aim to develop biomarkers for the genus and species-level diagnosis of bacteria using immunofluorescently-labeled lectins. Additionally, we aim to develop techniques suitable for fluorescent microscopes, which can be considered a faster and easier method for sensitive diagnosis.

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