Original Article

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Serological survey to detect antigens that cross-react with COVID-19 antibodies

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ABSTRACT

Background: COVID-19 first emerged in Wuhan City and has since spread worldwide, infecting over 600 million people. The World Health Organization (WHO) issued an emergency in public health in January 2020 due to reports of new coronaviruses in Europe, Asia, and the Americas. **Aim**: The study aims to screen bacterial antigens for cross-reactivity with COVID-19 antibodies using indirect ELISA and to detect cross-reactivity between vaccinated and unvaccinated healthy individuals and various bacterial types (*Gammaproteobacteria, Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, and Enterobacteria, in addition to Staphylococcus aureus*) either sonicated or non-sonicated. **Methods**: ELISA is a method for detecting unknown samples in a sample. It involves pipetting 50 µl of diluted antigen dilution into PVC microtiter plates, with pure antigen test samples pipetted at 2 µg/ml. The plate is incubated at room temperature for 2 hours or overnight at 4°C. Blocking is done with 200 µl of blocking buffer. The plate is then incubated overnight at 4°C or room temperature for 2 hours. Primary and secondary antibody incubation is performed, with two hours of room-temperature incubation and overnight incubation for stronger staining. Absorbance is measured for each well, and the results can be analyzed using a standard curve. **Results**: Results showed that vaccinated individuals had highly significant p-values of IgG antibodies against *S. aureus, Pseudomonas aeruginosa, Enterobacteria, and Bacillus*, while unvaccinated individuals had non-significant p values. **Conclusions**: The high cross-reactivity identified *E. coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterobacteria,* and *Gammaproteobacteria*. The current study was validated by the discovery of a significant correlation between Pfizer vaccinated individual's serum antibodies and unvaccinated individual's serum and the examined bacterial antigens that had a high titer of cross-reactivity with the vaccinated samples in comparison to the unvaccinated samples (less titer).

Keywords: COVID-19, ELISA, bacterial antigen, vaccinated and unvaccinated individuals.

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INTRODUCTION

Coronavirus was first encountered globally in 2002 due to Severe Acute Respiratory Syndrome (SARS), then in 2011 due to Middle East Respiratory Syndrome (MERS). Both instances were caused by newly found coronaviruses of zoonotic origin belonging to the genus Beta-coronavirus.[1] The currently prevalent COVID-19

made its debut in Wuhan, China, in late 2019. Human-to-human transmission occurs due to intimate contact,[2] and those infected with COVID-19 develop severe respiratory disease and quickly spread over the globe.[3,4] More than 6.5 million individuals have died due to the COVID-19 illness, which has infected over 600 million people [World Health Organization (WHO) up to November 25, 2022].[5]

The molecular structure of COVID-19 has four key proteins: the spike (S), envelope (E), membrane (M), and nucleocapsid (N). COVID-19 shares approximately 80% of its genetic sequence with SARS-CoV, the virus that caused the SARS pandemic in 2002.[6]

Despite the similarities, the S protein of COVID-19, which enables the virus to connect to the angiotensin-converting enzyme (ACE2) receptor, is many amino acids longer than the SARS-CoV S protein. This may be why COVID-19 has expanded so rapidly over the globe, in contrast to SARS-CoV, which was immediately restricted to its original location.[7] Human angiotensin-converting enzyme 2 (ACE2) is a type I membrane protein that binds viral S proteins. COVID-19 binds to ACE2 10 to 20-fold more strongly than SARS-CoV, which may explain why human-to-human transfer is simpler.[8]

Approximately 80% of patients had a moderate illness; 14% had significant signs or symptoms, such as shortness of breath, hypoxia, or lung infiltrates (parenchyma) and had indications suggestive of severe illness about 5%, including shocks, respiratory failure, or multiple organ failure, while the total mortality rate for the group was 2.3%.[9]

COVID-19 symptoms vary from person to person. Some infected people do not develop any symptoms (asymptomatic). Other people infected with COVID-19 are considered symptomatic patients based on the following symptoms: fever or chills, cough, shortness of breath or difficulty breathing, tiredness, muscle or body aches, headaches, loss of taste or smell, sore throat, congestion or runny nose, nausea or vomiting, and diarrhea. Symptoms may appear 2–14 days after exposure to the virus. Children have similar but usually milder symptoms than adults.[10,11] Transmission can occur by coughing, sneezing, and inhaling droplets, as well as direct contact with the mouth, nose, and eyes. The most common transmission routes between humans are through direct contact, droplet contamination, and the air.[12,13]

There are two main types of diagnostic procedures available for COVID-19: those that look for the actual COVID-19 and those that look for antibodies to that virus. Virus detection tests typically employ techniques like reverse-transcriptase polymerase chain reaction (RT-PCR) and isothermal amplification, which recognize and amplify SARS-CoV-2 viral nucleic acids. The SARS-CoV-2 virus is typically tested in a dedicated laboratory using respiratory samples like nasopharyngeal swabs, though near-patient tests have also been developed. Tests for detecting SARS-CoV-2 antibodies, also known as serology testing, can be performed on blood or serum samples and have been optimized for use in both a laboratory setting and a "near-patient" setting.

Serology tests may help identify a current, recovering (convalescent), or past SARS-CoV-2 infection because antibodies are produced as part of the immune response to infection[14] like ELISA is a method for measuring antigen-antibody binding that can be applied to the quantification of peptide, protein, antibody, and hormone levels.[15]

Yalow and Berson first described antibody-mediated detection linked to a radioactive signal (radioimmunoassay) in 1960. In light of this information, alternatives to radioactivity were investigated. When it was found that specific enzyme-substrate combinations caused measurable color changes, immunodetection took a turn. An antibody-enzyme substrate combination was developed to detect a narrow range of analytes.[16]

When using the ELISA method, antigens are first immobilized on a solid surface, where they are subsequently bound with antibodies to form an antigen-antibody bond complex, which is then bound to the enzyme. The reaction between the enzyme and the substrate will produce the detection signal, which takes the form of a color change.[17] This study used bacterial antigens like *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacteria, Staphylococcus aureus, and Bacillus*.

Several other probiotics, like Acidophilus®, Enterogermina®, Probiotic-10®, Probiogen®, and Meda-Probiotics®, were also used. Both probiotics and bacterial antigens are suspected to produce antibodies similar to those produced by the spike protein COVID-19 and may also be stimulators for the immune system.

Vaccine development has been sped up to obtain immunity to the virus and put an end to transmission as many countries continue to fight off infections caused by COVID-19.[18,19] Usually, making a vaccine takes a long time and is a lot of work. Recent progress in making a vaccine against COVID-19 has shown that research

advances build on what is already known and add to it. This makes it possible for the COVID-19 vaccines to be made quickly enough to stop a pandemic. Vaccines are biological mixtures that give you active immunity to a certain infectious disease. They do this by making the immune system react to a molecule on the pathogen called an antigen.[20] Vaccines have been around since the time of Edward Jenner, who made the first one for smallpox in 1796.[21]

MATERIALS AND METHODS

Study Design and Settings

Between December 2021 and April 2022, a cross-sectional observational study was carried out. The study was conducted at Basra Teaching Hospital, Basra Governorate, Iraq.

The ethical and scientific committee of the faculty of pharmacy at Basra University, in addition to the scientific committee of research at the Basra Health Directorate, approved this study.

Administrative in Addition to Ethics-Related Factors

A research proposal was presented to the university committee outlining the study's goals and the procedures that would be used to collect data, as required by the graduate studies section of the College of Pharmacy at Basra University.

After agreeing, the proposal for the current study was sent to the Basra Health Directorate's committee for ethical approval. The committee of the mentioned center gave its approval.

Patient's Selection

Only one hundred patients, N = 100 (60 males and 40 females), who consented to the study were included. All samples were taken from healthy people who were only vaccinated with the Pfizer vaccine (about 80) and unvaccinated (about 20). The main inclusion criteria are as follows: The age range of patients was 18–90 years of either sex. Patients and relatives should be able to communicate and be willing to participate in this study, including patients who were vaccinated with Pfizer only. The exclusion criteria are patients with acute infectious diseases like pneumonia and the human immunodeficiency virus and patients who have been vaccinated with any type of vaccine except Pfizer.

Inclusion Criteria

1) The ranged age of patients was 18–90 years of either sex.
2) Patients and relatives should be able to communicate and be willing to participate in this study.
3) Patients who were vaccinated with Pfizer only.

Exclusion Criteria

1) Patients with acute infectious diseases like pneumonia and the human immunodeficiency virus.
2) Patients who had been vaccinated with any type of vaccine except Pfizer.

Serum test

After the serum is taken from the vaccinated patient, it must be tested to detect the presence of IgG or IgM, according to manufacturing data.

It was unzipped and ready to use right before testing began. Blood samples stored in the refrigerator were brought back to room temperature, generally between 15°C and 30°C, before being used in the analysis. A 10 µL serum sample or 20 µL of whole blood sample was taken using a pipette, 2–3 drops (approximately 70–100 µL) of PBS buffer were added to the sample port to drive capillary action along the strip, and the entire exam was completed in about 15 minutes.

Bacterial Strains

Several bacterial strains are used to detect cross-reactions between these bacteria and the IgG of a vaccinated patient; all types are identified by using VITEK 2 in the College of Pharmacy as shown in Table 1.

Table 1: Bacterial isolate and Supplier Origin

No.	Strain of Bacteria	Supplier
1	*Staphylococcus aureus*	College of Pharmacy, University of Basra
2	*Pseudomonas aeruginosa*	College of Pharmacy, University of Basra
3	*Escherichia coli*	SADR Teaching Hospital
4	*Klebsiella pneumoniae*	College of Pharmacy, University of Basra
5	*Bacillus*	SADR Teaching Hospital
6	*Enterobacteria*	SADR Teaching Hospital

ELISA Techniques

In this procedure using indirect ELISA subtype both buffers and reagents, refer to the protocol.[22] Quantitative results are most reliable when the signal of an unknown sample is compared to a standard curve.

Each plate must be run alongside a standard (in duplicate or triplicate) and a blank.[23]

The general procedure for ELISA was followed. A sterile 96-well plate was taken, and the antigen was coated on the microplate with PBS. Another carbonate buffer can also be used. The buffer can be used to dilute the antigen to a final concentration of 20 µg/ml. The antigen dilution (50 µl) was pipetted into the top wells of a PVC microtiter plate to coat the wells. The samples were watered down when necessary (pure antigen test samples are typically pipetted onto the plate at a concentration of 2 µg/ml). While it is not necessary to have perfectly pure solutions, it is recommended that the target protein make up more than 3% of the protein in the sample being tested (antigen). The microtiter plate was overloaded with more than 20 µg/ml of antigen protein, which caused most of the sites to become saturated. The plate was then incubated for 2 hours at room temperature or 4°C overnight after being covered with adhesive plastic. The incubation time for the coating could be improved, and after filling the wells with 200 µl of PBS solution, the coating was removed, and the plate was washed three times. The plate was flipped over a sink to drain the solutions or washes. The plate was dabbed with a paper towel until all the moisture was absorbed.

The next process is blocking, where 200 µl of blocking buffer (5% non-fat dry milk or 5% serum in PBS) was added to each well to block the remaining protein-binding sites in the coated wells. Block ACE and BSA are two other potential blocking reagents. After incubating the plate for at least 2 hours at room temperature (or overnight at 4°C), it was covered with adhesive plastic and placed in the refrigerator. Then, two cycles of PBS dishwashing were completed.

Incubation in the next step, where diluted primary antibodies (100 µl) were added to each well, was then incubated for 2 hours at room temperature, and the plate was covered with adhesive plastic. Although 2 hours are typically sufficient to obtain a strong signal, stronger staining is often observed when incubated overnight at 4°C. If a weak signal is obtained after four PBS washes of the plate, then the manufacturer-recommended concentration of conjugated secondary antibody could be added to 100 µl of blocking buffer just before use. After 1–2 hours of room-temperature incubation, the plate can be covered with adhesive plastic and placed in the refrigerator. Then, the wells were washed four times with PBS, 100 µl of the substrate solution was pipetted into each well, and 100 µl of stop solution was added. Finally, an ELISA plate reader was used to measure the absorbance (optical density) at wavelength 450 nm.

RESULTS

ELISA results for bacterial antigen cross-reactivity between vaccinated individuals and unvaccinated individuals

Sonicated and non-sonicated bacterial antigens and vaccinated individuals

The bacterial antigens used in this study (*E. coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterobacterias,* and *Gammaproteobacteria*) were taken and subjected to the sonication process. The samples can be divided into two groups: sonicated and non-sonicated. The statistical analysis t-test was used, and the results are shown in Fig. 1. When sonicated and non-sonicated bacterial antigens were compared at an optical density of 450 nm, we observed a p value of 0.0023 for non-sonicated *Staphylococcus aureus* samples, which can be considered highly significant, and non-sonicated *Enterobacteria and E. coli* samples had significant p values of 0.0237 and 0.0241, respectively, but other bacterial antigens, *P. aeruginosa, Bacillus, and Klebsiella*, had non-significant p-values of 0.1379, 0.882, and 0.0822, respectively. Each star symbol stands for a significant value.

Sonicated bacteria and unvaccinated individuals

The serum samples of unvaccinated healthy individuals were taken and checked for reactions with several dilutions until 1/2000 of bacterial antigens previously prepared using the sonication process, as shown in Fig 1. The results of a one-way ANOVA of several bacterial antigens (*Pseudomonas aeruginosa, S. aureus, E. coli, Klebsiella, Bacillus,* and *Enterobacteria*) with a P-value of 0.0001 are shown in Fig. 2. Every bacteria must have a different symbol, like a (*P. aeruginosa*) and b (*S. aureus*) have significant P value, while cd (*Bacillus*) is not significant P value when compared with c (*E. coli*), d (*Klebsiella*), or d (*Enterobacteria*).

Sonicated and non-sonicated probiotics and vaccinated individuals

The main aim of this study is to determine whether taking probiotics orally can stimulate the immune system in a manner comparable to that induced by COVID-19. Several probiotics, including Acidophilus, Biolact, Probiotics-10, Enterogermina, Meda cap, and Pro-B,

were evaluated to achieve this. Both Acidophilus and Biolact had significant p values of 0.0243 and 0.0492, respectively. Each star symbol represents a significant value. While the other probiotics, Probiotics-10, Enterogermina, Meda cap, and Pro-B, have non-significant p values of 0.8399, 0.2613, 0.3758, and 0.49, respectively, as shown in Fig. 3.

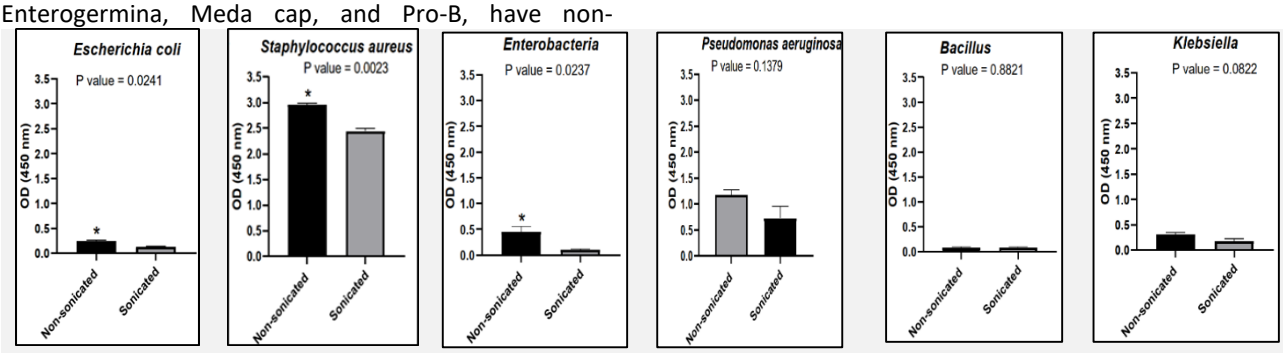

Figure 1: Sonicated and non-sonicated *S. aureus, E. coli, Enterobacteria, Bacillus, P. aeruginosa, and Klebsiella* and samples from vaccinated individuals.

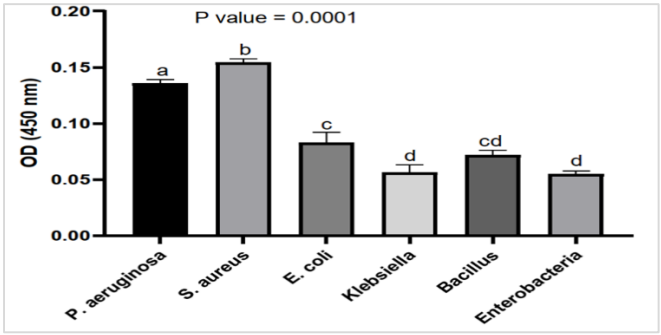

Figure 2: Sonicated bacterial antigens and samples from unvaccinated individuals.

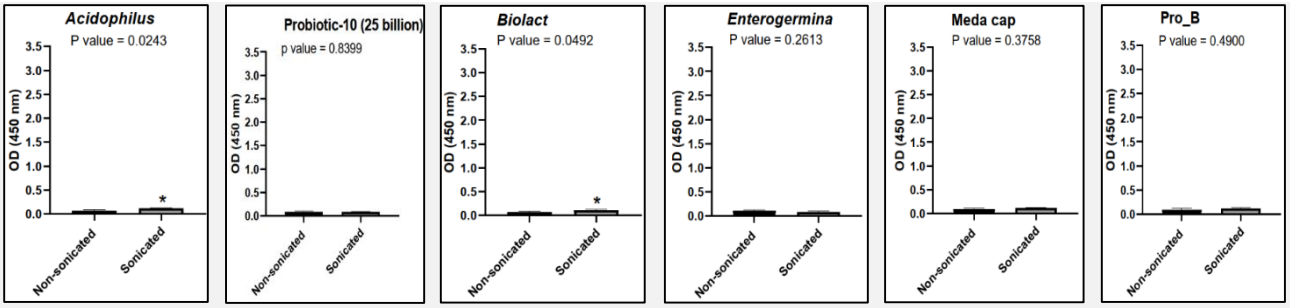

Figure 3: Sonicated and non-sonicated probiotics with samples from vaccinated individuals.

DISCUSSION

Since it was identified in late 2019, COVID-19 has been a major cause for concern for public health worldwide. It causes severe cases of acute respiratory syndrome and typically infects the lower respiratory system. Due to its rapid spread and high mortality rate, the current outbreak of COVID-19 has been declared a global emergency, so developing and using a vaccine could be crucial in either eradicating the virus from human populations or controlling its spread within communities if strict and thorough measures are taken. There have been significant advances in terms of how we learn about the origins and development of viruses, prompting numerous efforts to deal with the problem that has just appeared.[24,25]

The serum of vaccinated and unvaccinated individuals was tested using ELISA for cross-reactivity with bacterial proteins and probiotics. This study uses indirect ELISA techniques to find bacterial antigens and probiotics that stimulate the immune system into producing IgG and IgM like that in individuals infected with COVID-19 or vaccinated individuals with mRNA vaccine (Pfizer vaccine). Here, serum from vaccinated individuals acts as antibodies and reacts with bacterial antigens and probiotics, and the same process is repeated for serum from unvaccinated individuals. The results show that the vaccinated samples have more reactivity to most bacterial antigens. This is because vaccinated individuals have immunoglobulin (IgG) that binds with bacterial antigens and, when read by an ELISA reader, gives a high OD compared to serum from unvaccinated individuals. This result is similar to Root-Bernstein's (2022) finding that human blood proteins have similarities to bacterial proteins. This has been linked to co-infections with COVID-19. Antibodies have been shown to react with multiple proteins in the blood that are produced due to similarities between bacterial proteins and proteins in human blood. Most of their antibodies could identify proteins from the bacteria *Streptococcus, K. pneumoniae, Staphylococcus, and Escherichia coli*.[26]

Additionally, there is an ongoing search for another route of administration for immunization, like oral or nasal administration. Since SARS-CoV-2 infections persist even among the vaccinated, there is an urgent need for a different vaccine platform to overcome the major limitations of the current COVID-19 vaccines. It needs to be less expensive, readily scalable, and easier to store and administer. Preclinical and clinical studies have shown that oral immunization has been shown to have many benefits compared to other routes for mucosal immunization. These benefits include enhanced stimulation of lymphoid tissue in the digestive tract, improved mucosal immune system response, decreased risk of infection, decreased expenses, increased production of IgA as an anti-viral simplification of dosing for human or animal use, and antigen access toward a larger mucosal area. The activation of dendritic cells has been shown to be significantly higher after an oral vaccination than vaccination from the nasal route. Increased production of CD8+ T cells, protection from virus provocation in vivo, and secretory IgA specificity are all achieved.

This theory is supported by in vivo research showing that ingesting recombinant Lactic acid bacteria through the mouth rather than injecting it directly into the abdomen results in higher levels of neutralizing antibody activity. The intranasal route of immunization is less effective than oral immunization with recombinant Lactobacillus in stimulating the production of neutralizing antibodies in the respiratory system, especially secretory IgA.

This study, similar to Giovanni Sarnelli's (2023) study done in mice, showed that through plasmid transfection, the commonly used apathogenic *E. coli* (Nissle strain) could be induced to express and expose the SARS-CoV-2 spike protein on the bacterial surface, suggesting a new immunization strategy.[27]

CONCLUSIONS

The present study concludes the following:

1. High cross-reactivity was observed with *E. coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterobacteria, and Gammaproteobacteria*.
2. The current study was validated by the discovery of a significant correlation between Pfizer vaccinated person serum antibodies and unvaccinated person serum with examined bacterial isolate antigens that had a high titer of cross-reactivity with the vaccinated person in comparison to the unvaccinated person (less titer).

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