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EnzyPha, An Engineered Helper Phage Developed to Overcome most of the Limitations Regarding Phage Titration and ELISA Tests

Faezeh Fouladvand [1], Farid Azizi Jalilian [2], Peyman Bemani [3], Masoud Saidijam [4], Razieh Amini [1*] ID

[1] Department of Molecular Medicine and Genetic, Faculty of Medicine, Hamedan University of Medical Sciences, Hamedan, Iran
[2] Department of Virology, Faculty of Medicine, Hamedan University of Medical Sciences, Hamedan, Iran
[3] Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
[4] Research Center for Molecular Medicine, Hamedan University of Medical Sciences, Hamedan, Iran

Corresponding Author: Razieh Amini, PhD, Associate Professor, Department of Molecular Medicine and Genetic, Faculty of Medicine, Hamedan University of Medical Sciences, Hamedan, Iran. Tel: +98-9167379116, E-mail: aminra14@gmail.com

Abstract
Introduction: The phage display method is a technology that enables the expression of exogenous polypeptides on the surface of bacteriophage particles. Phage titration and ELISA are applied to measure helper phage particles or polypeptide bearing phages and also evaluation the interaction between polypeptide bearing phages and coated antigens, respectively. Although several procedures have been introduced to perform phage titration and ELISA, they face some limitations, such as being time-consuming, expensive, and low reproducibility.
Materials and Methods: We developed a new system called EnzyPha by engineering the M13KO7 expressing Secreted Acid Phosphatase of *Mycobacterium tuberculosis* (SapM enzyme) on its pIX protein for applying in colorimetric phage titration and ELISA methods. To evaluate the idea, colorimetric phage titration and ELISA were performed and compared to the traditional methods.
Results: SapM enzyme was expressed on the pIX protein of M13KO7 properly. The colorimetric phage titration and phage ELISA showed better and comparable results against the traditional approaches.
Conclusions: The results showed that the proposed model would titrate phages more sensitively than the plating titration method through a shorter timeframe. Moreover, it could be a better alternative to the routine phage ELISA due to time-saving, cost-effectiveness, and higher sensitivity.
Keywords: Phage Display, Helper Phage, Phage Titration, Phage ELISA
Citation: Mohammadi M, Fouladvand F, Azizi Jalilian F, Bemani P, Saidijam M, Amini R. EnzyPha, An Engineered Helper Phage Developed to Overcome most of the Limitations Regarding Phage Titration and ELISA Tests. J Appl Biotechnol Rep. 2023;10(1):888-94. doi: 10.30491/JABR.2021.305741.1446

Introduction

Filamentous phages are rod-like shape semi-flexible viruses that infect a large number of gram-negative bacteria.[1,2] Structurally, these bacteriophages contain a circular single-stranded DNA (ssDNA) genome packaged into a tube-shaped capsid.[1] This tube comprises thousands of copies of main coat protein (pVIII) subunits capped at the phage's two ends by only a few copies of minor cots proteins (pIII/pVI at the proximal end and pVII/pIX at the distal end).[1]

The phage display method is a technology that enables the expression of exogenous polypeptides on the surface of bacteriophage particles.[3,4] In this method, the gene encoding a polypeptide of interest is inserted into the gene of one of the phage coat proteins, displaying the polypeptide on the surface of phage.[3] Depending on the purpose and the used phage display vector system, all five coat proteins, pIII, pVI, pVII, pVIII, and pIX, can be used as platforms for expressing proteins on the phage. Selecting the suitable coat protein relies on the size, amino acid composition, and the required valency of exogenous binder.[5,6]

In order to assess the efficacy of the panning process and determine the binding capability of finally isolated single clone binders, the resultant polyclonal phage pools derived from each round of panning and the individual single clone binders from the last round are evaluated by phage ELISA.[7,8] While phage ELISA is a routine and standard method for evaluating the binding ability of phage binders, it faces several limitations. Due to the need for several incubations and washing steps, this method is lengthy, tedious, time-consuming, and arduous,[8,9] and is expensive due to using a monoclonal antibody. Additionally, using antibodies (primary or secondary) lead to one or two extra layers on the phage binders, which is associated with its technical drawbacks, including false positive reactions because of non-specific interaction of antibody with polystyrene plate surface (due to

incomplete blocking or excessive washing).[10]

On the other hand, given the widespread applications of phage display, determining the titer of the phages is crucial for phage experiments. For example, to monitor panning progress and to normalize the phage titer of individual monoclonal phage binders for comparing their binding ability, there is a need to determine the phage titer.[8,11] The most common method quantitating phage is plating, method in which susceptible bacterial cells are infected with a dilution series of the phage and then plated on selective antibiotic-containing agar. After overnight incubation, the concentration of phage is determined by direct counting colony-forming units (cfu) or (plaque-forming units (pfu).[8,10] Though this method is the conventional and gold standard technique for quantifying phage, it suffers from some limitations; plating method is laborious and time-consuming.[8,12] Also, the assay relies on indirect detection of phage by evaluating its bacterial infectivity , which does not reflect the actual count of phage particles, especially when the phage titer is very high.[12] Other issues such as ion content and concentration, agar thickness and concentration, and bacterial health may affect phage titration and thus reduce the reproducibility of this method.[13-15]

In addition to the plating methods, other methods are used to measure the M13 phages number. Since phage structure is composed of nucleic acid and protein, the components that absorb ultraviolet (UV) light, UV spectrophotometry at a wavelength of 269 nm is another standard method for measuring phage concentration.[16-19] Although UV photometry are a simple and rapid method compared to plating, protein impurities from the culture supernatant that interfere with the UV absorption is the main weakness.[20] This forces the need for using highly pure phage that can be obtained by two subsequent PEG precipitations,[16,20] CsCl density-gradient ultracentrifugation[20] or chromatography,[21,22] which all make the method time-consuming, laborious, and expensive.

Recently, real-time qPCR has been developed as an alternative to the traditional phage titration assays.[23-25] While real-time qPCR is a sensitive method for phage quantification, it has some limitations; the genome of phage is used as starting material for PCR reaction, and this imposes the necessity of phage genomic isolation from the phage particles using a DNA extraction kit.[24,26] In addition, free phage DNA (non-encapsidated DNA) in the phage solution can mimic intact phage particles; thus, phage treatment with DNase is needed to remove false positive signals.[23-25] Furthermore, the PCR reaction is sensitive to the presence of PCR inhibitors and in cases that cannot be removed, this method is unreliable.[24]

According to the abovementioned, there is necessary to develop a new method for quantifying and titrating phage particles circumventing the drawbacks related to the other methods. In this work based on the our previous in silico study,[27] we aimed to engineer a helper phage (M13KO7) expressing SapM enzyme (Secreted Acid Phosphatase of *Mycobacterium tuberculosis*) on its pIX protein (Named EnzyPha system) for application in helper phage ELISA and titration methods. pIX is the minor coat protein expressed in five copies at the distal end of filamentous phage with only 33 amino acids.[1,2] Also, SapM is a mycobacterial acid phosphatase that functions in a wide range from acidic to neutral pH and exploits many organic phosphoesters as substrates.[28,29]

Materials and Methods
Preparation of M13KO7 Double-stranded DNA
E. coli TG1 bacteria which carries F´ plasmid, were cultured onto a 2TY plate containing 50 μg/ml kanamycin, 2% glucose and 5 mM $MgCl_2$ and incubated overnight at 37 °C. One ml of 2YT medium in a 1.5 ml sterile microtube was inoculated with a single colony from the agar plate. The culture was incubated for 2 hours at 37 °C in a shaker incubator. After reaching the optical density to 0.5, M13KO7 helper phage stock was added to the medium. The microtube was incubated at 37 °C for 30 min followed by shaking at 37 °C for 30 min. The infected bacteria were grown overnight at 37 °C on 2TYG plates containing antibiotics. A single colony was inoculated in 5 ml 2TY broth and incubated at 37 °C overnight with 180 rpm shaking. Bacteria suspension was centrifuged at 8000 rpm for 5 min. The supernatant was poured out, and the bacterial pellet was applied to isolate the helper phage double-stranded DNA using a mini prep plasmid extraction kit (Qiagen, USA).

Cloning of SapM Sequence into the M13KO7 Genome
Purified double-stranded DNA of helper phage and PUC57 vector containing the SapM sequence (NC_000962.3) were digested (double-digestion) separately with *Bsr*GI and *Sna*BI restriction enzymes (Fermentas, Lithuania). Subsequently, the SapM sequence was ligated into the M13KO7 DNA using T4 ligase (Fermentas, Lithuania) based on the company's instruction. Finally, the ligation mixture was transformed by the heat-shock $CaCl_2$ method into the *E. coli* TG1 strain.

Helper Phage Particle Concentration
Released recombinant helper phages were precipitated by the PEG-NaCl method. Briefly, 50 ml media containing recombinant M13KO7 were clarified for 15 min by centrifugation at 14,000 g at 4 °C, and the supernatants were decanted. Helper phage was precipitated from the clarified supernatant by adding 20% (w/v) PEG 6,000 and 2.5M NaCl and incubated on ice for at least one hour. Precipitated M13KO7 particles were concentrated by centrifugation at 3,300 g for 45 min at 4 °C and then re-suspended in 1 ml PBS. Recombinant helper phages were filtered through a

0.22-μm filter membrane (Millipore, USA).

Recombinant Helper Phage Titration
Traditional Approach

Phage titration was done by infecting *E. coli* TG1 with serially diluted helper phages (recombinant helper phages). Briefly, 100-fold serial dilutions of the concentrated helper phage were prepared in PBS. Afterward, TG1 cells (OD600 reached approx. 0.6) were infected with the diluted phages by incubating for 30 min without shaking and then 30 min with shaking at 37 °C and 200 rpm. Finally, the infected bacterial cells were plated on 2TYG containing 50 μg/ml kanamycin overnight at 37 °C.

Colorimetric Approach

150 μl of each serial diluted recombinant helper phage were transferred into the 96-well plates., 150 μl of pNPP substrate solution (5 mM p-nitrophenyl phosphate, 0.1% Triton X-100 and 0.1M sodium acetate [pH 5.0]) was added to each well. The plates were incubated at 37 °C for 30 min. Subsequently, the reaction was stopped by adding 10 μl of 1 N NaOH, and color changing was assayed at 405 nm using an ELISA reader.[30] As the negative control, non-enzymatic hydrolysis of the substrate was determined by including wells that contained non-recombinant helper phages. All experiments were performed in triplicate.

Phage ELISA

200 μl of recombinant and non-recombinant M13 helper phages (10^8 cfu/ml in PBS) were coated on 96 well plates separately and placed overnight at 4 °C temperature. Afterward, the wells containing the phages were washed three times with PBS/Tween 20 (0.05%) and five times with PBS. 200 μl of BSA 2% in PBS was added to the wells as a blocking solution for two hours at room temperature. Following washing with PBS, for recombinant phage, 150 μl of pNPP substrate (5 mM p-nitrophenyl phosphate (pNPP), 0.1% Triton X-100 and 0.1 M sodium acetate [pH 5.0]) was added. The plates were incubated at 37 °C for 30 min. Then the reaction was stopped by adding 10 μl of 1 N NaOH, and color changing was assayed at 405nm using an ELISA reader.[30] Also, for non-recombinant phage, 150 μl of anti-M13 bacteriophage HRP conjugated (Sigma, UK) (1:20000 in blocking solution) was added to each well and incubated for one hour at room temperature. Subsequently, the plate was rinsed and stained using 100 μl TMB buffer and placed in the dark at room temperature for 30 min. Finally, the reaction was stopped with H_2SO_4, and each well's optical density (OD) was read at 450nm using an ELISA reader.

Statistical Analysis

Differences between groups were determined based on an unpaired t-test. Data analyses were performed using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, CA).

Results
Cloning of SapM Sequence into the M13KO7 Genome

Two enzymes, BsrGI and SnaBI, were used for cloning of SapM gene into the M13KO7 genome (8668 bp). BsrGI cuts gene VII at nucleotide 1022, and SnaBI cuts gene IX at nucleotide 1271. The former enzyme creates a sticky end, while SnaBI generates a blunt end with no nucleotide overhang. Upstream of the SapM sequence, a g3 signal sequence was placed to conduct the recombinant construct within periplasmic space and ensure proper integration of SapM-pIX into the helper phage framework during phage assembly. An 849 nucleotides fragment (798 nucleotides related to the SapM and 51 nucleotides related to the g3 signal peptide genes) was situated upstream of the pIX gene (Figure 1). The eliminated sequences belong to the pVII and pIX genes by acting the two restriction enzymes, were added before g3 signal peptide and after SapM sequences, respectively. Eventually, 266 amino acids regarding the SapM enzyme will be expressed on the pIX subunit of the M13KO7 helper phage.
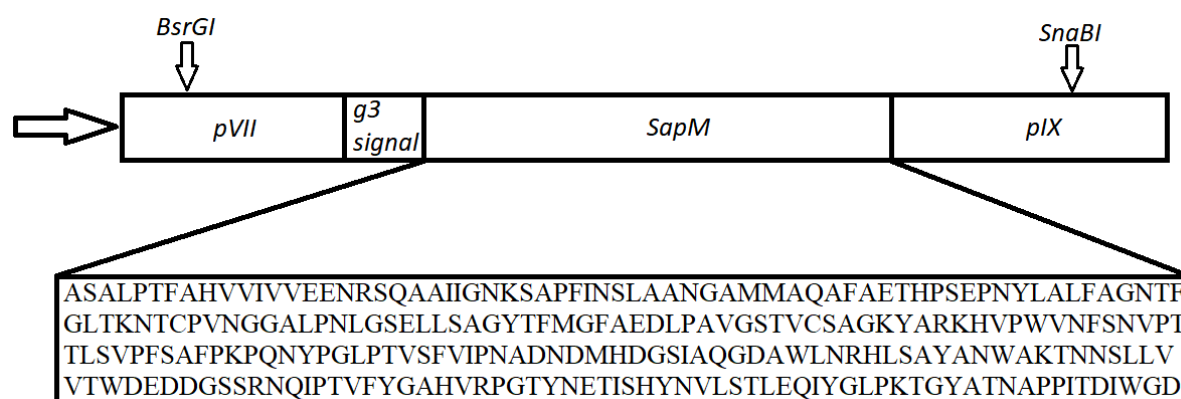

**Figure 1.** The EnzyPha Construct and SapM Amino Acids Sequence.

Phage Titration

The achieved titers by plating and colorimetric methods were 10 and 12-fold dilutions of phages, respectively. Also, there is a linear relationship between different dilutions and absorption graphs for the EnzyPha system. Moreover, minimum absorbance was observed for the same dilutions of the non-recombinant helper phages as a negative control in 30 min. As shown in figure 3, the colorimetric method was detected an insufficient number of phage particles, approximately 50 phages/ml. In contrast, the minimum limit of detection by the traditional method was about 10^3 phages per ml (data not shown).

Phage ELISA

After 30 min, the difference between the color of reactions regarding the EnzyPha model and M13K07 as the negative control reached to maximum. Furthermore, the EnzyPha system significantly showed a higher mean absorbance value compared to the traditional phage ELISA test (anti-M13 antibody) (Figure 3). Although, the optical absorptions obtained at two different wavelengths for two methods, but difference between the mean absorbance of the test and control were more significant for engineered helper phage ($p<0.0001$) than traditional phage ELISA ($p = 0.0003$) based on unpaired t-test.
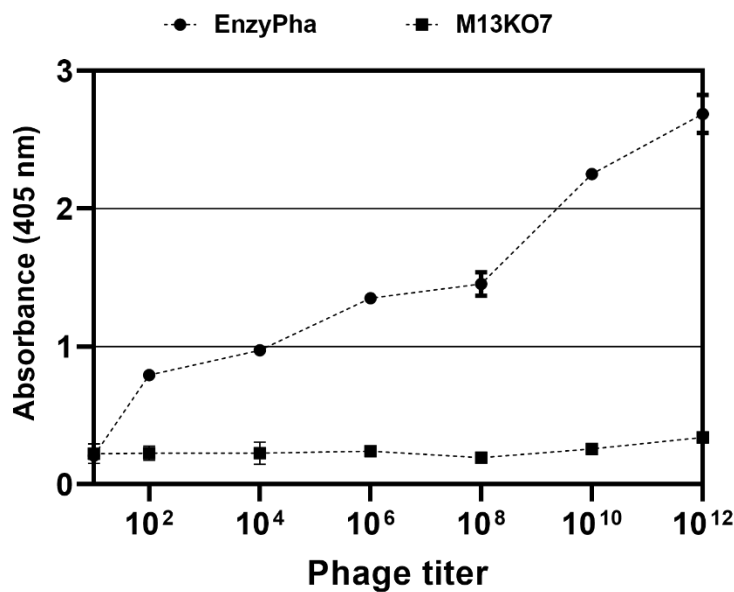
Figure 2. Colorimetric Phage Titration Plot. Optical absorptions were measured for each dilution regarding the recombinant helper phage (EnzyPha) and non-recombinant M13KO7 (negative control) by ELISA reader.
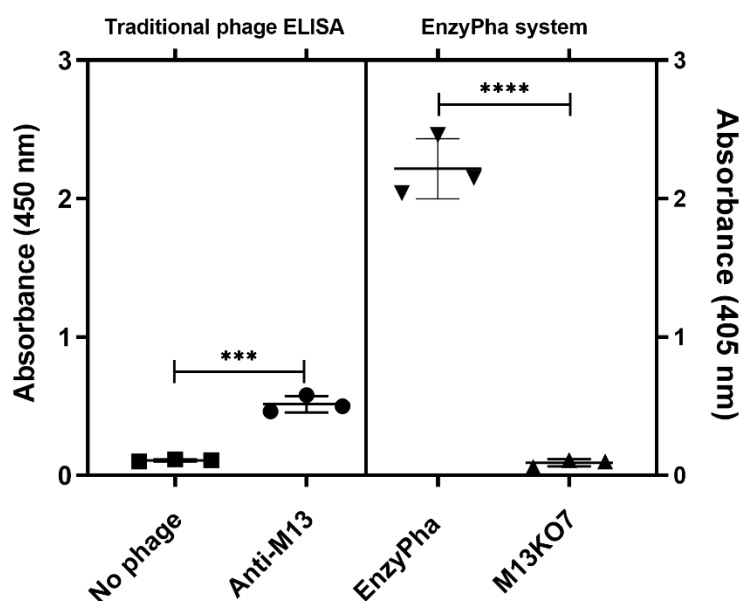

Figure 3. Phage ELISA Results. For two methods, tests were compared to the negative controls. For traditional method, no phages were coated in the wells and non-recombinant M13KO7 was used as the negative control for EnzyPha system. Created colors at the end of the tests were measured at 450 nm for traditional phage ELISA and 405 nm for EnzyPha method. *$p<0.05$ is based on unpaired t-test.

Discussion

Phage display is a valuable method with various applications in microbiology, immunology, biotechnology, and nano-technology. Titration of the isolated phage particles is one of the requirements for the phage display technique. On the other hand, phage ELISA test is performed when the phage display method is used to evaluate ligand-receptor or antibody-antigen interactions. In this work, we introduced the EnzyPha system in which SapM enzyme is expressed on the pIX protein of M13K07 helper phage (Figure 4). We aimed to overcome several limitations related to the phage ELISA and phage titration assays.
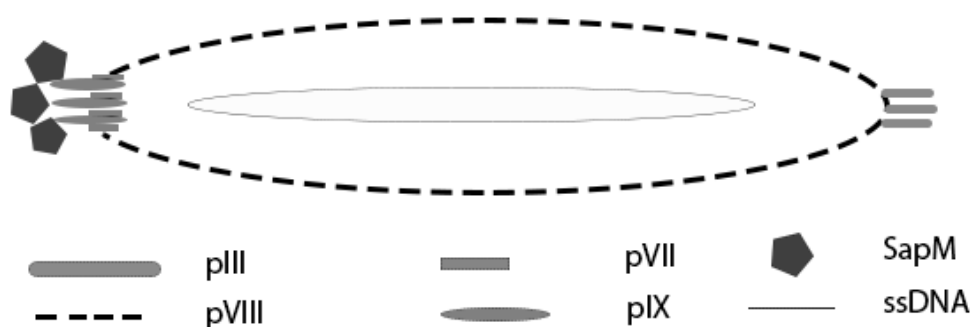
Figure 4. Schematic Representation of SapM Expressing M13KO7 Helper Phage (EnzyPha).

So far, various methods have been proposed for phage titration. However, the gold standard method counts the phage particles by providing a serial dilution and culture on a plate after bacterial infection (plating method).[31] The plating method may only sometimes provide accurate results for several reasons, including a multiplicity of infection (MOI), which is the ratio of phages to bacteria in the culture medium where phages attach to the bacteria and infect them.[32] Some phages cannot infect bacteria for various reasons, such as inappropriate pili, temperature, or culture medium conditions.[33] On the other hand, some bacteria are infected by more than one phage simultaneously. Unfavorable results may also obtain because of bacterial contamination.

In the colorimetric titration method (EnzyPha system), serial dilutions of the recombinant M13KO7 phages are also provided without infecting bacteria. Indeed, the phage numbers are directly measured in the buffer by detecting the intensity of the color generated through the enzymatic reaction. Consequently, the MOI phenomenon would not interfere with this procedure The proposed procedure is to demonstrate the actual titer of phages. Also, the EnzyPha method showed a wider detection range than the gold standard plating method. Regarding the upper detection limit, it could detect 100-fold higher phage particles than that obtained from the gold standard method. Regarding lower detection limit, it was more than 10-fold more sensitive.

Moreover, the non-enzymatic hydrolysis of the substrate for negative control at various dilutions was trivial and insignificant. In this respect, Yang and colleagues have shown that non-enzymatic hydrolysis of pNPP may be delayed at least up to 2 hours.[30] However, in addition to pNPP, other compounds such as α-Naphthyl phosphate and Phosphoenolpyruvate are also hydrolyzed by SapM enzyme with 100% activity However, pNPP is more recommended according to its activity in broader pH values.[29]

The engineered helper phage can also be a good alternative to the routine phage ELISA technique. PhoPhabs was an attempt to overcome the disadvantages of the traditional phage ELISA introduced by Light and Lerner in 1992.[34] They expressed the bacterial alkaline phosphatase on the pVIII subunit of the VCSM13 helper phage. In that work, they used a secondary vector (pPho8cat) and the antibody-bearing phagemid to express the alkaline phosphatase. Unlike them, we constantly expressed SapM enzyme on the helper phage's pIX protein. Variations in the number of the expressing enzymes on the helper phages could affect the repeatability of their results. In addition, the presence of relatively large and homo dimer enzymes, such as alkaline phosphatase on the pVIII protein, the major coat protein of M13 helper phage could inhibit the assembling of all phage subunits and consequently decrease the phage yield. In the case of expressing the SapM enzyme on the pIX subunit, the proper distance will be kept between the enzyme and pIII subunit, which usually is used for displaying exogenous polypeptides. Therefore, they will not interfere with the function of each other.

Differences between the absorbance of the test and negative controls were more significant for the proposed method than traditional phage ELISA. It made our method more sensitive than traditional phage ELISA where there are samples with low antigen values or low copy number of phages. Moreover, in the routine phage ELISA, several harsh washing could lead to the loss of manyanti-M13 or secondary antibodies. However, in the new method, the generated color directly correlated to the number of phages. In this study, all experiments were performed in triplicate,

and low standard deviation (SD) values indicated the repeatability and reliability of the results regarding the EnzyPha system.

As expected, the engineered helper phage would be applied in the routine phage display, especially in the panning process,[35] where the phages displaying antibodies or other polypeptides encounter the immunotubes or ELISA well plates coated antigens. At this stage, phages that show low or no affinity to the antigen are removed by washing, and the attached phages are recycled and enriched by infecting *E. coli* bacteria. Using the proposed model, the number of phages bound to the coated antigen can be assessed in less than 30 minutes before infecting the bacteria. In this case, the success rate would be predicted at every step, and consequently, waste of time and materials would be avoided.

Finally, as a perspective, it is expected that the engineered helper phage to be used in immunohistochemistry (IHC) and western blotting techniques using an appropriate substrate. Other enzymes with phosphatase and peroxidase activity would be expressed on the pIX subunit, and other M13 derivative helper phages would be manipulated similarly.

Conclusion

In the present study, we designed and developed a good alternative for M13KO7 helper phage and its derivatives, named the EnzyPha system, to overcome some trouble shooting regarding phage titration and ELISA methods. For this purpose, the SapM enzyme was expressed on the pIX protein of M13KO7. The results showed that the proposed model would titrate helper phages alone or, when used in phage display, more sensitive than the plating titration method throughout less time. Moreover, it could be a better alternative to the routine phage ELISA due to ti time-saving, cost-effective, and higher sensitivity.

Authors' Contributions

MM presented the idea for the article and writing and also critically revised the work. FF and PB performed the literature search and writing. All other authors contributed equally to this study.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

Acknowledgment

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