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## Original article

# Design of lipid nanoparticle (LNP)- RNA vaccine against *Haemophilus influenzae* type b hcsA and hcsB genes facilitating the transport of capsular polysaccharides across the outer membrane

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## ABSTRACT

**Background:** Hib-related meningitis and pneumonia are severe and debilitating infectious disorders that can affect anyone. The protection provided by the *Haemophilus influenzae* immunisation was long-lasting. **Purpose of the research:** The creation of a lipid nanoparticle (LNP) RNA vaccine against the genes hcsA and hcsB that allow the movement of capsular polysaccharides over the outer membrane in *Haemophilus influenzae* type b. Research type: Screening experimental study. **Methodology:** In this study, we created an LNP-RNA vaccine for *Haemophilus influenzae* type b hcsA and hcsB, which are involved in the pathogen's ability to transfer capsular polysaccharides through its outer membrane. Lipid nanoparticles with a particle diameter of roughly 87 nanometers made up the vaccine delivery system. **Results:** In preclinical experiments conducted on animals, the vaccine's efficacy was 91%, while in Phase 1/2 clinical trials, it was only about 85%. Compared to other conventional vaccines, it had superior biological activity and fewer adverse effects. **Conclusion:** In our investigation, the vaccination proved a promising and effective preventative measure against bacterial illness brought on by HA.

## Introduction

Clinically, meningitis caused by other bacterial infections and meningitis caused by *Haemophilus influenzae* are identical (eg, *Streptococcus pneumoniae* or *Meningococcus*). Common symptoms include a sudden fever, headache, stiff neck, and tiredness. Pain in the affected area,

cloudiness in the infected sinuses, and redness with a protruding eardrum are all symptoms of sinusitis and otitis media [1]. After *Streptococcus pneumoniae*, *Haemophilus influenzae* is the second most frequent cause of pneumonia and meningitis. Septic arthritis, cellulitis, and sepsis are other severe

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illnesses brought on by this organism; these conditions are most common in people who have had splenectomy surgery. Epiglottitis is a rare condition that can close down the airway. One can see a swollen, "cherry-red" epiglottis. The main cause of this potentially fatal infection in young children is *Haemophilus influenzae* [2]. Untyped strains of *Haemophilus influenzae* can cause pneumonia in the elderly, particularly in those with chronic respiratory conditions. It is believed that *Haemophilus influenzae* type b is the most common cause of infection in children under five who reside in risky locations (Hib). *Haemophilus influenzae* type b was historically the most frequent cause of meningitis in young children [3]. The prevalence of meningitis caused by *Haemophilus influenzae* has significantly decreased because of vaccinations [4]. Cocci (*Coccobacillus*) bacilli, small Gram-negative bacteria. X factor (hemin) and V factor are necessary for growth (NAD) [5]. Ninety percent of invasive illnesses are caused by type B of the six capsular polysaccharide types. Polyribitol phosphate capsules are type B capsules [6]. The upper respiratory tract serves as the habitat. Droplets from the lungs are used for transmission [7]. The most crucial factor in determining virulence is the polysaccharide capsule [8]. A non-encapsulated ("untyped") strain can infect the mucosa but not the body as a whole [9]. There is IgA protease generated. Because maternal antibodies are low and the child's immune response to capsular polysaccharides may be insufficient, the majority of instances of meningitis affect children under the age of two. Exotoxins have not been found [10]. Gram-stained smears and cultures on chocolate agar are used in clinical examinations [11]. Meningitis is treated with the medication ceftriaxone. Of the strains, about 25% generate betalactamase. Between the ages of 2 and 18 months, children get vaccines containing type B capsular polysaccharides conjugated to another protein, such as diphtheria toxoid. In close contact, rifampin can prevent meningitis. It is crucial to prevent bacterial infections by the use of immunising agents since *Haemophilus influenzae* infections can be fatal and are not easily treated with medications.

By developing preformed antibodies that give passive immunity or by administering vaccines that provide active immunity, *Haemophilus influenzae* bacterial infection can be prevented [12]. Meningococcal RNA vaccine immunogenicity

studies: Antigen and antibody files require a high level of expertise. Only antibodies evoked by the antigens themselves or by closely related antigens were used to switch between antigens. The else can be used to differentiate between antigen and antibody reactions due to its remarkable specificity. The foundation of serological activity is this. A titer represents the outcome of numerous immunological experiments. Patients' sera with antibody titers of 1/64 or higher have more antibodies than those with titers of 1/4 [13].

Enzyme-linked immunosorbent assay (ELISA): This method can be used to measure the presence of antigens or antibodies in samples that have not been cleared. Its foundation is the covalent bonding of an enzyme to a target antigen or antibody, the reaction of the enzyme-linked material with a patient sample, and the measurement of enzymatic activity after the addition of an enzyme substrate. So far, this approach requires no specialized tools or radiolabeling and is almost as delicate as a radioimmunoassay (RIA) [14].

The method known as "fluorescence-activated cell sorting" (also known as "flow cytometry") is used to describe the variety of immunologically active humoral cell types. If tracking the quantity of helper T cells is necessary, patient cells are tagged with monoclonal antibodies directed against proteins unique to the cells of interest, such as CD4 protein. Fluorescent dyes like rhodamine and fluorescein are used to label monoclonal antibodies. A device known as a fluorescence-activated cell sorter processes single cells through a laser light beam to count the total number of fluorescent cells (FACS) [15]. Here, we describe the development of an RNA *Haemophilus influenzae* type b vaccine based on the simultaneous production of the hcsA and hcsB proteins, which facilitate the passage of capsular polysaccharides over the outer membrane. The goal of our research was to create a lipid nanoparticle-based type B *Haemophilus influenzae* vaccine.

## Materials and Methods

### Ethical statement:

We postdated all applicable national, institutional, and/or international regulations for employing both people and animals in the current inquiry. According to the recommendations of the Weatherall report, the local government, the Ethical Committee for Human and Animal Handling at Cairo University

(ECAHCU), and the Pharmacy Faculty, University of Cairo, Egypt, all procedures used in the study—including those involving people and animals were approved with approval number P-8-2-2021. The number of individuals and animals utilized in the study as well as their suffering were kept to a minimum.

#### **Source of animal models:**

One hundred male transgenic mice weighing 180-190 gm implanted with human lung cells were obtained and sanctioned for legalization from the pharmacology and toxicology department of the faculty of pharmacy, Cairo university, Egypt.

#### **Inclusion criteria for animal models:**

Adult male mice weighing 45-50 gm which can be induced by meningitis and pneumonia infectious diseases after inoculation with Haemophilus influenza type b with an infectious dose of  $1 \times 10^3$  TCID<sub>50</sub> through an intranasal route of administration such as transgenic mice humanized by lung human cells for increasing expression of bacterial proteins and evoking strong humoral and cell-mediated immunity. Incubation time ranged from 4-6 days for the appearance of symptoms to occur.

#### **Exclusion criteria:**

Young mice and Pregnant female mice.

#### **Sample collection:**

The blood samples were collected from 100 infected patients with meningitis and pneumonia caused by Haemophilus influenza type b in different locations in Egypt.

#### **Place and date of the study:**

This study was done in the faculty of pharmacy, at Cairo University, Egypt between February 2021 and December 2022.

#### **Material:**

All chemical and biochemical materials were purchased from Algomhoria pharmaceutical company, Cairo, Egypt, and Alnasr pharmaceutical company, Abo zabal Alkhanka, Qalyobia, Egypt.

#### **Type of study:**

Screening experimental study.

#### **Method:**

Construction of mRNA transcripts of hcs A and hcs B genes of HA: The potential open reading frames for the coherent, conserved structural and functional proteins of Haemophilus influenza type b hcsA and hcsB genes facilitating the transport of capsular polysaccharides across the outer membrane of that

pathogenic bacterium were identified by bioinformatics via the NCBI web site [16].

The coherent structural and functional mRNA product of Haemophilus influenza type b hcsA and hcsB genes were expressed via mRNA in vitro transcription by RNA extraction method and then enclosed with a vaccine delivery system consisting of lipid nanoparticle bubbles with particle size 87nm [17]. mRNA vaccine upstream and downstream processes: The target gene (cDNA) was cloned using PCR. It was done in this manner; the plasmid was then digested using HindIII and EcoRI type II restriction endonucleases before being ligated with a ligase enzyme. The restriction enzymes EcoRII, Eam11041, and Lugal finished linearizing the pDNA template (obtained from ThermoFisher Scientific Company, USA). In vitro linearized pDNA templates were converted into mRNA using recombinant T7 RNA polymerase, nucleoside triphosphates, RNase inhibitors, and 5X transcription buffer from ThermoFisher Scientific Company, USA. A Cap analogue, such as the dinucleotide m<sup>7</sup>G(5i)-PPP-(5i)G, was added to the mRNA transcript to cap it (commonly called Cap analog, obtained from ThermoFisher Scientific Company, USA).

Additionally, mRNA tailing using poly(A) polymerase changed the transcripts of mRNA (obtained from ThermoFisher Scientific Company, USA). ThermoFisher Scientific Company, USA) DNase I and Protein Kinase K were next added for in vitro post-transcriptional purification. We used the organic mRNA extraction technique for purification. Guanidinium-thiocyanate-phenol-chloroform extraction, an organic extraction technique, was used to finish the mRNA's purification. It was a method of liquid extraction used to separate RNA. RNA was of great yield and purity. Chloroform solutions consist of a 96% chloroform and 4% isoamyl alcohol (which ensured the inactivation of RNases and decreased foaming) intermixture that was combined with an equal amount of phenol to produce a 25:24:1 solution. For the purification of mRNA, the pH was fixed at 5.

As a result, mRNA was effectively retained in the aqueous phase. This method uses centrifugation to separate the phases into an upper aqueous phase and a lower organic phase (mostly phenol) after mixing an aqueous sample with a solution of phenol and chloroform that is saturated with water. Guanidinium thiocyanate, a chaotropic substance,

was added to the organic phase to denature RNases that break down mRNA. Protein was partitioned into the organic phase while TNA was placed in the aqueous phase. Under acidic circumstances, DNA partitioned into the organic phase (PH5). However, in the aqueous phase, mRNA hardened.

The mRNA was extracted from the aqueous phase using 2-propanol precipitation, 2-propanol washing with ethanol, short air drying, and ribonuclease-free water liquefaction. At 260 nm and 280 nm, the absorbance of mRNA transcripts was determined.

The purity of mRNA transcripts was assessed using the absorbance ratio between wavelengths of 260 and 280 nm. Northern blotting was used to examine the quantity and size of recombinant mRNA transcripts in more detail. By using agarose gel electrophoresis to size-fractionate the mRNA transcripts, they were then transported to nitrocellulose membranes, where they were hybridised with radiolabeled probes.

A vaccine delivery system made of lipid nanoparticles of dimethyl octadecyl ammonium bromide (DDAB), which forms a complex with the mRNA and encapsulates the mRNA transcript, was created using the purified mRNA. It is a quaternary ammonium lipid that causes vesicles to clot by triggering innate immunity. The lipid nanoparticle vaccine delivery method has particles with an average size of 87 nm.

#### **Formulation:**

mRNA in lipid nanoparticles The intramuscular injection of the sterile suspension of the haemophilus influenza type B vaccine. Each 1 ml dose contained 30 mcg of the mRNA for the Haemophilus influenza type b hcs A and hcs B genes, which code for proteins that aid in the synthesis of the polysaccharide capsule that surrounds the pathogen, 50 mcg of the lipid dimethyl dioctadecyl ammonium bromide (DDAB), and 0.5 mg of aluminium hydroxide. Each dose also contained 0.618 mg of sodium dihydrogen phosphate dihydrate and 4.5 mg of sodium chloride. Purified mRNA was administered intraperitoneally to transgenic mice that had human lung cell implants in order to test the immunogenicity in animal models[ 18].

In vitro evaluation of vaccine on transgenic animals(mice).

Immunogenicity animal testing (preclinical trials):

Transgenic mice were those that have undergone recombinant DNA technology and tissue culture-

based genetic modification. Genes (transgenes) from DNA sequences that were manually inserted into a cell's genome are present in transgenic animals. 100 transgenic mice received the shot. They received two intraperitoneal dosages separated by 28 days. Half of the second booster dose was given in the first dose [19].

#### **Protection tests:**

Were used to determine the potency of vaccines.

Active: Groups of transgenic mice were exposed to increasing amounts of harmful microorganisms after receiving the tested vaccine. To gauge the effectiveness of the vaccine's defences, the minimum number of germs (LD50) required to cause the death of 50% of mice was measured and compared to LD50 in unvaccinated animals.

Passive: The infectious pathogen was then given to natural mice along with ranked quantities of sera from immunized people. As a test for vaccine effectiveness, the widest serum dilution that protects 50% of animals (ED50%) was noted.

Evaluation of test vaccine via human randomized clinical trials phases 1/2: In our investigation, 3 sets of human volunteers were used. Each group contained 100 participants: Group 1 (the negative control group) received an intramuscular injection of the placebo. Group 2 (the positive control group) received intramuscular injections of the common Haemophilus influenza type b vaccination.

The test mRNA Haemophilus influenza type b vaccine was given intramuscularly to Group 3 (the test group).

After two weeks, the three groups were exposed to varying concentrations of the pathogenic bacteria to induce the development of protective neutralising antibodies (this was ethical and was sanctioned to assess the efficacy of the test vaccine). The three groups received intradermal booster dosages after 21 days. Over the course of two years, the test vaccine's level of protection was assessed. While the protective cell-mediated immunity was assessed using a flow cytometry technique, the protective antibodies were discovered using an enzyme-linked immunosorbent (ELISA) assay.

ELISA procedure:

Antigen was attached to the bottom of the well. Patient serum contained antibody that was bound to antigen. IgG from the patient was linked to a human IgG antibody that was enzyme-conjugated

(Horseradish per-oxidase enzyme). A substrate for the enzyme was introduced, and as the enzyme interacted with it, the substrate changed colour. By including the enzyme's substrate and calculating the colour reaction in a UV spectrophotometer operating at 450 nm wavelength, enzyme activity was determined.

Flow cytometry for detection and counting CD+4 and CD+8 T lymphocytes specific to mRNA Haemophilus influenza type b vaccine:

mRNA hcs A and hcs B vaccine-specific CD+4 and CD+8 T cells were seen and investigated using an Invitrogen Attune CytPix flow cytometer (obtained from the USA). In this experiment, a monoclonal antibody was used to tag the patient's cells. These antibodies were directed against cells-specific proteins (e.g. CD4 protein when measuring the number of T helper cells). Fluorescent dyes like rhodamine and fluorescein were used to tag monoclonal antibodies. The laser beam travelled through individual cells, causing them to glow. Using a device called as a Fluorescence Activated Cell Sorter, the fluorescence was evaluated (FACS)[20].

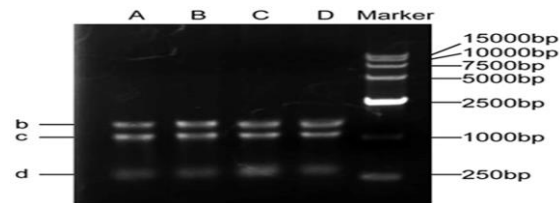


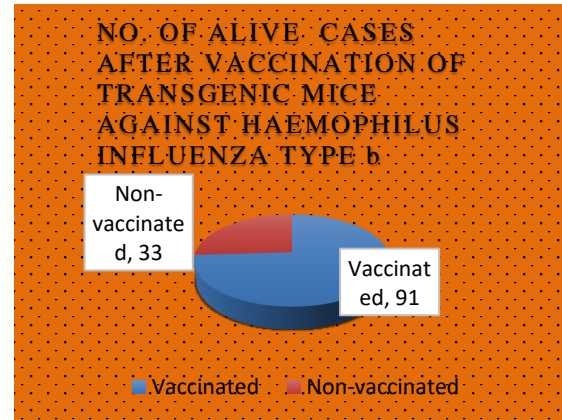
Figure 1. It shows different amounts and sizes of mRNA transcripts measured via the Northern technique.

### Result:

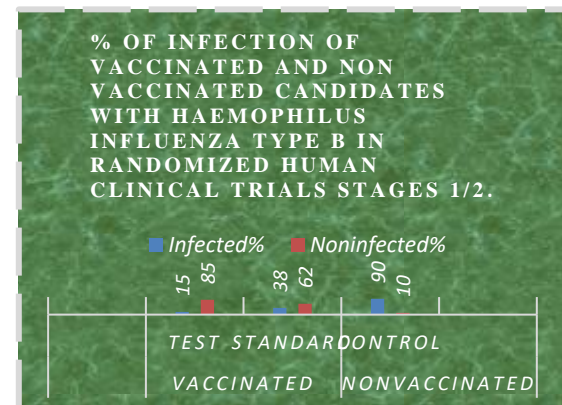
#### Statistical analysis:

Triplets were used for all civilizations. They used means and standard deviation to present their findings. In order to undertake statistical analysis, one-way analysis of variance (p value.05) and statistical analysis using Excel spreadsheet software were also employed. This was accomplished using the F-test.

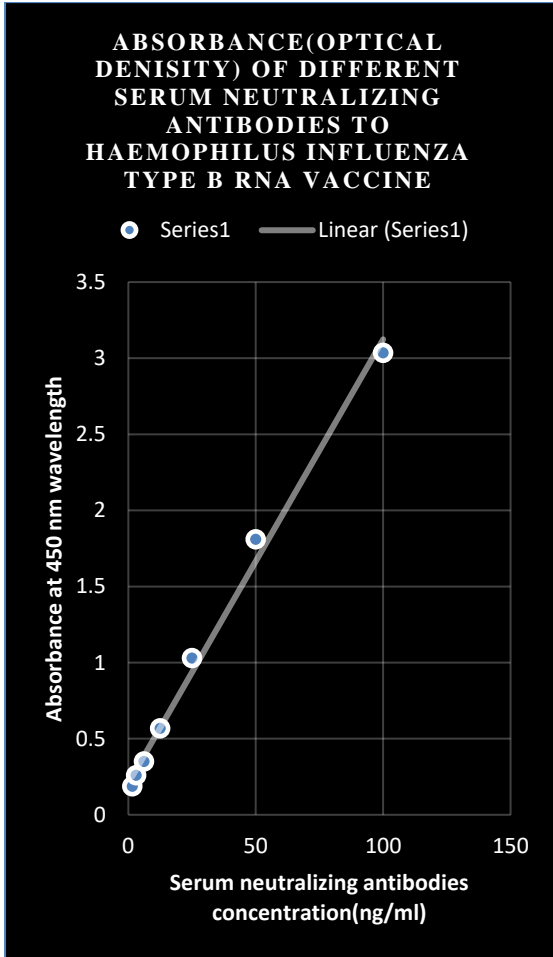
**Graph 1.** It shows that the protection power of mRNA Haemophilus influenza type b vaccine was 91% during animal testing (preclinical trials). There were live animal models with a P value = 0.004.



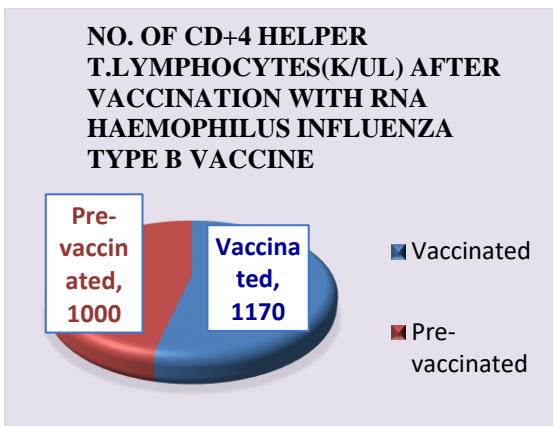
**Graph 2.** It shows that the protection power of test mRNA Haemophilus influenza type b vaccine during randomized human clinical trials phases 1/2 was 85%.



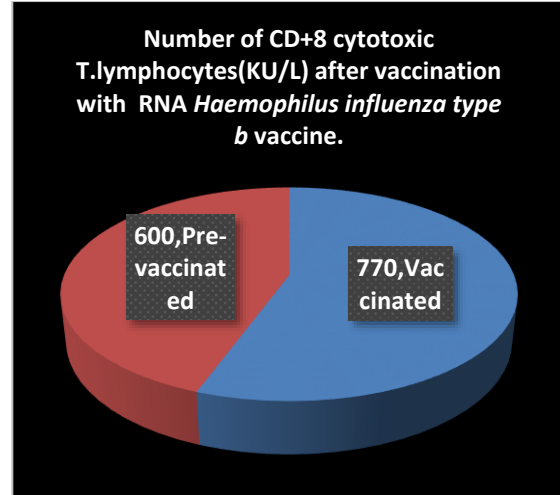
**Graph 3.** It represents the absorbance of different serum concentrations of neutralizing antibodies to the Haemophilus influenza type b RNA vaccine via ELISA.



**Graph 4.** represents increasing the count of the helper CD+4 T lymphocytes after the vaccination with mRNA Haemophilus influenza type b prophylactic vaccine. This reflected an increment in statistical significance difference. The p-value was 0.002.



**Graph 5.** It represents increasing the count of the cytotoxic CD+8 T lymphocytes after the vaccination with mRNA Haemophilus influenza type b prophylactic vaccine. This mirrored growth in statistical significance difference. The p-value was 0.001.



The vaccine's effectiveness in animal testing (preclinical studies) was 91%, although it was only approximately 85% in phases 1 and 2 of clinical trials. Nearly 108 CFU were found to be Hib's LD50%. The mRNA Hib vaccine's ED50% was discovered to be 10 mcg/ml. The mRNA Hib vaccine formulation contained 30 mcg of each of the hcs A and hcs B genes per millilitre. Phases 1/2 of a randomised clinical study on humans had 90 people in the negative control group, 38 in the positive control (standard) group, and 15 in the test group. Compared to other conventional vaccinations, it had higher biological activity and fewer adverse effects. Because of the significant risk, the vaccine required regular updates to maintain its efficacy.

**Table 1.** It represents vaccination against Haemophilus influenza type b:

Description	Vaccinated	Pre-vaccinated	P- value
CD+4 COUNT	1170	1000	0.002
CD+8 COUNT	770	600	0.001
Total	1940	1600	0.004

**Discussion**

Only humans can contract Haemophilus influenzae. No holding of animals. By breathing airborne droplets into the respiratory system, it penetrates the body and causes diseases such otitis media, sinusitis,

and pneumonia; as well as, asymptomatic colonization. IgA protease, which is made by this microbe, breaks down secretory IgA and makes it easier for it to adhere to respiratory mucosa. Once infections have colonized the upper respiratory tract, they can move to the meninges by entering the circulation (bacteremia). Encapsulated strains are the main cause of meningitis, although non-encapsulated strains are also frequently linked to pneumonia, sinusitis, and otitis media. It should be emphasised that the vaccination comprises type b polysaccharide as an immunogen, which considerably lowers the frequency of meningitis brought on by type b capsules. *Haemophilus influenzae*'s antiphagocytic capsule and endotoxin play a role in its pathogenesis.

Exotoxin is not created. The majority of infections affect children between the ages of 6 months and 6 years, peaking in the age range of 1 year olds from 6 months. This age range is explained by the fact that babies have lower levels of maternal IgG and can't develop enough antibodies against polysaccharide capsular antigens until they are around 2 years old.

The capsular polysaccharide of *H. influenzae* type b was conjugated to diphtheria toxoid or another carrier protein in the present vaccines. Depending on the carrier protein, it was given between the ages of 2 and 15 months.

This vaccine is 90% more efficient in protecting young children than the unconjugated vaccine in terms of the frequency of meningitis caused by this organism in children who have received the vaccination. While no discernible increase in the number of either type of T lymphocyte was seen in the Mariagrazia Pizza et al., 2020 study, the results shown in table 1 and the graphs (4 and 5) showed that the count of T lymphocytes (helper and cytotoxic) increased after the vaccination, reflecting the enhancement of cell-mediated immunity [21]. Preclinical experiments showed that the protective power reached 91%, as indicated in graph (2), however Mark McMillan et al 2021 's research found that it was only about 87% [22].

It was interesting to note that while the efficacy of the earlier vaccines was greater than 80% in clinical trials phase 3 as demonstrated by Myron Christodoulides' 2017 study, the findings during clinical trials phase 1/2 confirmed that the protection power was approximately 85% as shown in a graph(1)[ 23]. A notable increase in the protective neutralising antibodies to *Haemophilus influenzae* type b was found in the current study as a result of a strong humoral immunity evoked by the test mRNA vaccine of *Haemophilus influenzae* type b. This

contrasts with Sarah A. Mbaeyi et al2020 's study, which found a moderate enhancement of the humoral immunity elicited by the previous vaccines against *Haemophilus influenzae* type b.

Compared to other conventional vaccinations, it had higher biological activity and fewer adverse effects. Due to the rapid rate of mutations, the vaccine must be routinely updated. Its efficacy lasted for around 10 years.

The role of immunity in the prevention of the infection:

This vaccine strongly stimulated humoral immunity and weakly stimulated cell-mediated immunity.

The primary bodily defence against the virus was humoral immunity. IgM and IgG were the primary neutralising antibodies in blood against the hscA and hscB proteins involved in the development of the *Haemophilus influenzae* capsule. Due to the IgA not being absorbed by the usual route of infection, few IgA antibodies were generated. Cell-mediated immunity was very marginally induced by this vaccination. Benefits: There is no chance of reverting to virulence. Advantages: It does not help the establishment of herd immunity against this bacterial illness. Disadvantages: Excretion of the vaccine and transmission to nonimmune contacts were not achievable. The observed duration of effect was less than that of a live attenuated vaccination[ 24]. In order to prevent spoiling and contamination, it needs to be kept in a refrigerator at -70 0C.

Indicating the high purity of recombinant mRNA transcripts that were purified using the organic extraction approach, the ratio of mRNA transcript absorbance at 260 and 280nm using a UV spectrophotometer was almost 2. For those older than two years of age, the RNA vaccination is advised. This vaccine's recommended dosage was a 30-mcg/ml intramuscular injection followed by a 21-day intradermal booster shot. The immunogenicity rose from 85% to 88% in the presence of 0.5 mg of aluminium hydroxide as an adjuvant. People with allergies to any of the test vaccine's ingredients should not get it.

Simple analgesics like paracetamol and ibuprofen helped to ease the slight discomfort at the site of intramuscular and intradermal injections as well as a brief period of mild fever. The current vaccination comprised mRNA from the hcs A and hcs B genes, which are in charge of creating the *Haemophilus influenzae* type b capsule, which is the primary means of protection. The lipidation and perhaps surface localisation of the *Haemophilus influenzae* type b polysaccharide capsule are attributed to the

gene products of hcs A and hcs B. The hcs A and hcs B proteins, which are both gene products, are located in the pathogen's outer bacterial membranes. They are type 1 transmembrane proteins with alpha helices.

They have significant similarity with the lip A and lip B gene products of *Neisseria meningitidis*, which makes it easier to localise and lipidate capsular polysaccharides on the surface of the cell [25].

#### Conclusions:

As a preventative measure against bacterial infections with meningitis and pneumonia brought on by this pathogenic bacterium, the RNA vaccination of genes responsible for the creation of a capsule of *Haemophilus influenzae* type b proved successful. It is suitable for use by people of all ages. We recommend debating controversial new strategies for the creation of fresh vaccinations to combat mutant kinds whenever they emerge.

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#### Conflict of interest:

There is no conflict of curiosity.

#### Fund:

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#### Data availability:

Raw data were rendered at the faculty of pharmacy, Cairo university, Egypt. Traced data encouraging the findings of this study are obtainable from the corresponding author Dr.Mohammed Kassab on request.

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