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Development of an Edible Vaccine Using Conserved PspA Epitopes Against Pneumococcal Infection in a Murine Model

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Abstract

Streptococcus pneumoniae, also known as pneumococcus, is one of the leading causes of respiratory tract infections, meningitis, and septicemia in Bangladesh. In recent years, the pneumococcal surface protein A (PspA) has been suggested as a promising candidate, alone or with other immunogens, to be included in a future vaccine. Edible plant-based vaccines use recombinant antigens produced in plant tissues, providing an affordable and oral delivery option compared to traditional vaccines. This study applied a computational reverse vaccinology strategy to identify four potential B-cell epitope peptides from the PspA protein. Four epitope peptide sequences were found as the most potential B cell epitopes. Conservancy analysis was done using *in silico* tools. The sequences of *S. pneumoniae* epitope sequences were analyzed and placed in fusion with alpha zein. *Agrobacterium tumefaciens* strain LBA4404 containing the Ti plasmid was used for selection in all cases for the integration of the gene of interest. Antigens of *S. pneumoniae* fused to zein in *Lycopersicon esculentum* were generated using *Agrobacterium*-mediated genetic transformation protocols. The transformation through *A. tumefaciens* was confirmed by kanamycin selection and PCR analysis with the specific primers. Mice immunized with transgenic plant tissue and the control mice were challenged intraperitoneally with 10 times the LD_{50} dose of the *S. pneumoniae* type 7F. The results demonstrated significant protection, with 83% of immunized

mice surviving the live S. pneumoniae challenge compared to only 50% survival in control groups. These findings highlight the immunogenic hotspot within PspA as a promising candidate for developing novel oral pneumococcal vaccines.

Key words: Edible vaccine, PspA, *Streptococcus pneumoniae,* Ti plasmid, T-DNA, Alpha zein, Gamma zein.

Introduction

Pneumococcal diseases remain a serious public health concern in developing countries, including Bangladesh. Streptococcus pneumoniae (Pneumococcus), encapsulated by a polysaccharide capsule, is responsible for significant morbidity and mortality worldwide [1, 2]. Pneumonia accounts for approximately 19% of child deaths globally, contributing to around 1 million deaths annually among children under 5 years between 2000 and 2003, with S. pneumoniae being a major contributor to this burden.

Pneumococcal surface protein A (PspA) is among the most prevalent surface molecules and a key determinant of protective immunity. Mutagenesis studies have demonstrated that PspA plays a critical role in nasopharyngeal colonization and virulence [3]. This choline-binding protein, ranging from 67 to 99 kDa in molecular size, exhibits high genetic variability and has been used to study the global distribution of pneumococci [4, 5]. Since the PspA gene is present in all S. pneumoniae isolates, it represents a promising vaccine target. PspA-based immunization has demonstrated protection against pneumococcal infection [6, 7]. As a cell wall-associated protein, PspA contributes to virulence by binding human lactoferrin and interfering with complement deposition on the bacterial surface [8, 9]. Computational approaches for epitope identification have advanced considerably over the past three decades [10].

Despite widespread antibiotic use, pneumococcal disease has not been eliminated. Although vaccines against S. pneumoniae have long been available, the rise of drug-resistant strains has become an increasing concern [11]. In plant-based vaccine systems, antigenic components are delivered into plant leaf cells using a non-pathogenic bacterial vector, prompting an immune response upon consumption. The plant then produces the target protein used for vaccination. Such systems offer cost-effectiveness and scalability, potentially expanding vaccine access to resource-

limited regions. Developing edible vaccines involves introducing selected antigen-encoding genes into plant or animal hosts, which subsequently synthesize the corresponding proteins — a process known as 'transformation,' yielding 'transgenic plants' or 'transgenic animals.' Like conventional subunit vaccines, edible vaccines contain antigenic proteins without pathogenic genetic material, making them safe even for immunocompromised individuals [12].

Agrobacterium tumefaciens-mediated transformation is one of the most widely used methods for introducing foreign genes into plant cells. A. tumefaciens is a gram-negative soil bacterium capable of inducing crown gall disease by transferring a segment of its DNA into host plant cells [13, 14]. This tissue culture-based approach typically yields primary putative transgenic plants within 4–5 months [15]. The Ti plasmid of A. tumefaciens facilitates this process by integrating a defined DNA segment, the T-DNA, into the host plant's chromosomal DNA. Transfer and processing of T-DNA are largely governed by virulence (vir) genes encoded on the Ti plasmid [16].

In this study, an edible vaccine was developed through Agrobacterium-mediated genetic transformation. Predicted B-cell epitope peptide sequences were analyzed, fused with zein protein, cloned into expression constructs, and used for plant transformation followed by immunogenicity evaluation.

Methods

The experiments of this research study were carried out in the Department of Microbiology, University of Dhaka and Genomic Research Laboratory, Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh. A total of 36 Swiss Albino mice were obtained from the Animal Resources Division, icddr,b, Dhaka and maintained in the Department of Microbiology, University of Dhaka, under hygienic condition and all animal experiments were done following the Ethical guidelines set the Faculty of Biological Sciences, University of Dhaka. To repeat the experiments (2^{nd} trial), same number of mice were obtained again. Therefore, a total of 72 Swiss Albino mice were used in this study, and all animal experiments were conducted in accordance with institutional ethical guidelines and approved animal welfare protocols.

Experimental Design

The experiment was conducted to develop a plant-based edible vaccine targeting *Streptococcus pneumoniae* using the candidate protein PspA, and the overall strategy has been illustrated in the figure 1. Initially, the structural gene encoding PspA was selected and cloned into a suitable plant expression vector. This recombinant vector was introduced into plants through *Agrobacterium tumefaciens*-mediated transformation. The selected experimental plants included *Lycopersicon esculentum* and *Nicotiana tabacum*, which were genetically modified to express the target antigen. After generating transgenic plants, the expressed protein was analyzed for immunogenicity using animal models such as rabbits and mice. The ultimate objective of this experiment was to evaluate the feasibility of using these transgenic plants to produce an edible vaccine, as represented by the final step in the figure where a child is shown consuming the plant-based vaccine product.

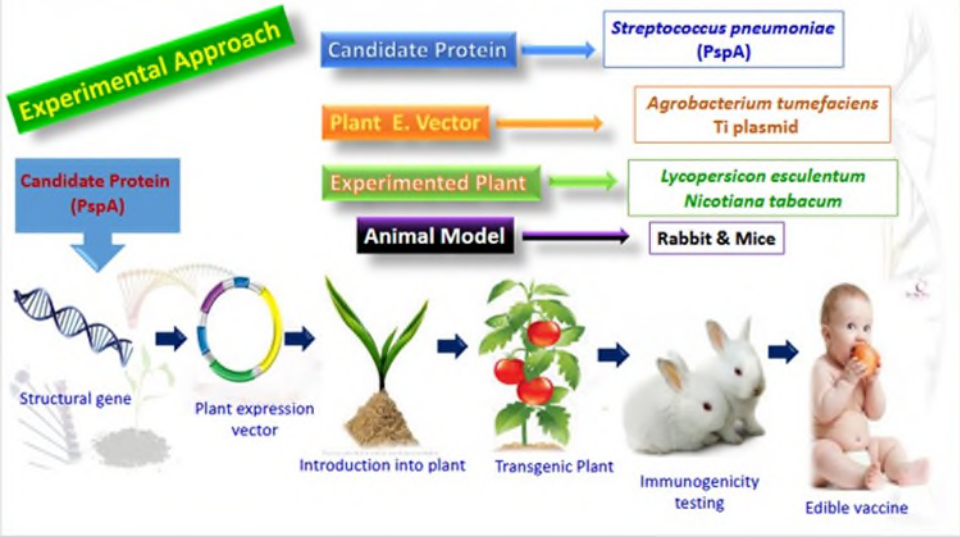


Fig. 1: Diagrammatic representation of the experimental design of development of an edible vaccine

Strain collection, maintenance, and identification

The *S. pneumoniae* serotype 7F strain, was maintained at -80°C in 12% glycerol at the Department of Microbiology, University of Dhaka. Working cultures were prepared by subculturing onto blood agar plates followed by overnight incubation at 37°C in a 5% CO_2 atmosphere. The organism was grown in brain heart infusion (BHI) broth supplemented with 10% fetal bovine serum (FBS) at 37°C for 24 hours on a shaker. However, the identity of these strains was reconfirmed by the Gram staining, optochin susceptibility, and bile solubility tests.

The bacterial strains were grown on gentamycin blood agar, and a colony was streaked onto a fresh blood agar plate. After the loop was streaked across the plate, an optochin disk (Ethylhydrocupreine hydrochloride, with a diameter of 6 mm, containing 5 g, BBL, USA) was placed aseptically at the end of the streak where the loop was first placed. The plates were incubated at 37°C for 24 hours, and a haemolytic growth greater than 14 mm in diameter was identified as *S. pneumoniae* [17].

B-cell epitope prediction of linear epitopes from protein sequence

Parameters such as hydrophilicity, flexibility, accessibility, and antigenic propensity of polypeptide chains have been correlated with the location of continuous epitopes in a few well-characterized proteins [18]. In this study, the Kolaskar and Emini antigenicity prediction method was employed for evaluating the potential of the peptides as linear B cell epitopes. The Kolaskar method was used to calculate the antigenic propensity (Ap) of residues by analyzing 156 antigenic determinants (20 amino acids) in 34 different proteins [19]. This method can predict the antigenic determinants with about 75% accuracy.

Another method followed in this study was the Emini method. The calculation was based on the surface accessibility scale on a product instead of an addition within the window. The accessibility profile was obtained using the formulae Sn = (n+4+i) (0.37) -6 Where Sn is the surface probability, dn is the fractional surface probability value, and i varies from 1 to 6 [20]. From these analyses, four predicted epitopes were selected on the basis of the frequency of their presence. These are: (i) ILTSLASVAILGAGFVASQPTVVRAEESPVASQ; (ii) AQKKYEDDQKKTEE; (iii) SESEDYA; (iv) SKAEKDYDAA.

Codon optimization

A synthetic polynucleotide sequence was designed and optimized for heterologous expression of PspA in plant systems. Following back-translation of the selected amino acid sequences, codon optimization was performed to match the codon usage bias of the expression host. For this reason, specific tables have usually been used. The nucleotides of respective peptide sequences, which have been obtained after considering the codon biasness of *S. pneumoniae,* are provided here. The selected peptides and nucleotide sequences are selected considering these two factors.

Preparation of the constructs

The sequences of *S. pneumoniae* epitopes have been introduced into a plant expression vector (pTRA) and transferred into *Agrobacterium tumefaciens*. Four predicted peptide sequences were selected and those were placed in fusion with alpha zein or the N-terminal 90 amino acids of the 27KD gamma zein, respectively. For easy detection, the alpha zein construct (construct alpha) contains a flag- and his-tag, the gamma zein construct (construct gamma) contains only the his-tag.

The constructs with alpha and gamma zein 1,2,3 and 4 are the sequences for the epitops of *Streptococcus pneumoniae*. The HIS- and the FLAG-Tag are important for the detection. NcoI, NotI and Xbal are restriction sites. The constructs are of different sizes. Construct gamma has about 500 bp, which corresponds to 18 kD. Construct alpha is twice the size of construct gamma (1000 bp or 36 kD). These constructs were applied to a vector which contained an *E. coli*-origin and a resistance gene for ampicillin. For multiplication, the constructs were re-transferred into *E. coli* Omni Max 2T1R, plated on LB-agar with ampicillin overnight, and incubated in 5 ml of LB-media with ampicillin. The next step was to purify the vectors using the QIAprep Spin Miniprep kit. After determining the concentration using NanoDrop, the vectors were digested with NcoI- and Xbal. The digestion mix was loaded on an agarose gel and separated at 70 V for 40 min.

Web application design for Primer

In this investigation, a new software tool called SnapGene viewer and Primer-BLAST were used to alleviate the difficulty in designing target-specific primers. This tool is sensitive enough to detect targets that have a significant number of mismatches to primers and combines BLAST with a global alignment algorithm to ensure a full primer-target alignment.

***Agrobacterium*-mediated genetic transformation and regeneration**

Different culture media were used in the present investigation for the *in vitro* regeneration protocol establishment of *Lycopersicon esculentum* (Tomato). For shoot initiation and their further development, MS medium supplemented with different combinations of hormones such as BAP (6-Benzyl aminopurine), Kn (Kinetin), NAA (-naphthalene acetic acid), IAA (Indole-3-acetic acid), and Zn (Zeatin) were used during the present experiment.

For the induction of roots at the base of the *in vitro* regenerated shoots, half strength of MS medium (half strength of macro, micro, organic nutrients, and iron source of MS medium) supplemented

with IAA (Indole-3 acetic acid), IBA (Indole-3 butyric acid), or NAA (-naphthalene acetic acid) were used. In this experiment, Gelrite (Duchefa, Netherlands) was used as the solidifying agent for the rooting media, whereas agar was used in all the other experiments.

Animal Ethics, Welfare, and Humane Endpoints

All animal experiments were conducted following the ethical guidelines of the Faculty of Biological Sciences, University of Dhaka, and approved by the Institutional Animal Care and Ethics Committee. Swiss Albino mice (36 per trial, two independent trials) were obtained from icddr,b, Dhaka, and maintained under standard laboratory conditions with free access to food and water. All animal experiments were conducted in strict accordance with relevant guidelines and regulations. All experimental protocols were reviewed and approved by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka, Dhaka, Bangladesh (Ref. No. 363/Biol. Scs./2025-2026, dated 01 March 2026). No human subjects were recruited or sampled in this study; the bacterial strain was obtained from an existing clinical repository with no direct human involvement. Therefore, informed consent from human participants is not applicable to this study.

Humane Endpoints:

To reduce suffering from pneumococcal infection, humane endpoints were predefined. Animals were monitored at least twice daily, and more frequently during the acute phase. Mice were euthanized if they showed >20% body weight loss, severe hypothermia, behavioral abnormalities (hunching, immobility, tremors, inability to access food/water), physiological distress (labored breathing, dehydration, unresponsiveness), or neurological signs (seizures, abnormal posture).

Minimizing Suffering:

Challenges were performed under light anesthesia (isoflurane). Mice were group-housed with enrichment and closely monitored. Analgesics were not used, as they could alter infection outcomes; this was approved by the Ethics Committee.

Challenge of mice with *S. pneumoniae* serotype 7F

The mice were grouped into numbers 1, 2, 3, and 4; each group consisted of twelve mice. Group 1 consisted of those mice that had been fed the transgenic plant that was integrated by the alpha

zein gene. Group 2 represented the mice that had been fed the transgenic plant integrated by the gamma zein gene. Group 3 included those mice that were fed by the non-transgenic plant. Different groups of mice with the transformed plants (alpha, and gamma) were each challenged with approximately 10^7 live cells of the virulent *S. pneumoniae* serotype 7F. This dose was ten times higher than the lethal dose (LD50). In each mouse, 0.50 ml of bacterial solution was injected intraperitoneally. Alive or dead, the mice were recorded daily for 30 days. Two trials were performed, named the 1st and 2nd trials, to confirm the results.

Euthanasia:

Animals were sacrificed by CO_2 inhalation followed by cervical dislocation, per AVMA Guidelines (2020).

Statistical analyses

All the tests are performed with SPSS in its version 22.0. The software name originally stood for Statistical Package for the Social Sciences (SPSS), reflecting the original market, although the software is now popular in other fields as well. The results were statistically analyzed to consider the variance of the mice challenge experiment with T-test, Oneway ANOVA, Post Hoc Tests etc.

Results:

Querying and linking the data of PspA

PspA is a highly prevalent surface-associated protein and a key target for protective immune responses against pneumococcal infection. The PspA sequences were compared against the GenBank database at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) using the Basic Local Alignment Search Tool (BLAST) to characterize different PspA genotypes.

Selected predicted peptide epitope sequences

Multiple B-cell linear epitope prediction algorithms are accessible through the IEDB platform. Among these, the Kolaskar-Tongaonkar antigenicity method and the Emini Surface Accessibility prediction tool are widely used and capable of predicting B-cell epitopes with approximately 75% accuracy. The Kolaskar-Tongaonkar algorithm identified a linear epitope spanning the 7–39 region. The 33-amino acid sequence at positions 7–39 was found to have the potential to elicit Tc,

Th, and B-cell responses. Accordingly, the first selected epitope sequence is: 1. ILTSLASVAILGAGFVASQPTVVRAEESPVASQ. Physicochemical parameters of polypeptide chains — including hydrophilicity, molecular flexibility, surface accessibility, and antigenic propensity — were evaluated to identify continuous epitopes within the PspA protein sequence. Based on Emini Surface Accessibility predictions, the remaining selected epitope peptides are: 2. AQKKYEDDQKKTEE; 3. SKAEKDYDAA; 4. SESEDYA.

PspA is a highly conserved protein

Multiple sequence alignment (MSA) conducted using MEGA 7.0 revealed that the predicted epitope peptide sequences are highly conserved across all analyzed PspA sequences, with no sequence variation observed among the collected and aligned protein sequences (Fig. 2). All four selected epitope peptides — ILTSLASVAILGAGFVASQPTVVRAEESPVASQ, AQKKYEDDQKKTEE, SKAEKDYDAA, and SESEDYA — were found to be perfectly matched across the complete set of PspA protein sequences examined (Fig. 2), confirming their conservation and suitability as vaccine targets.

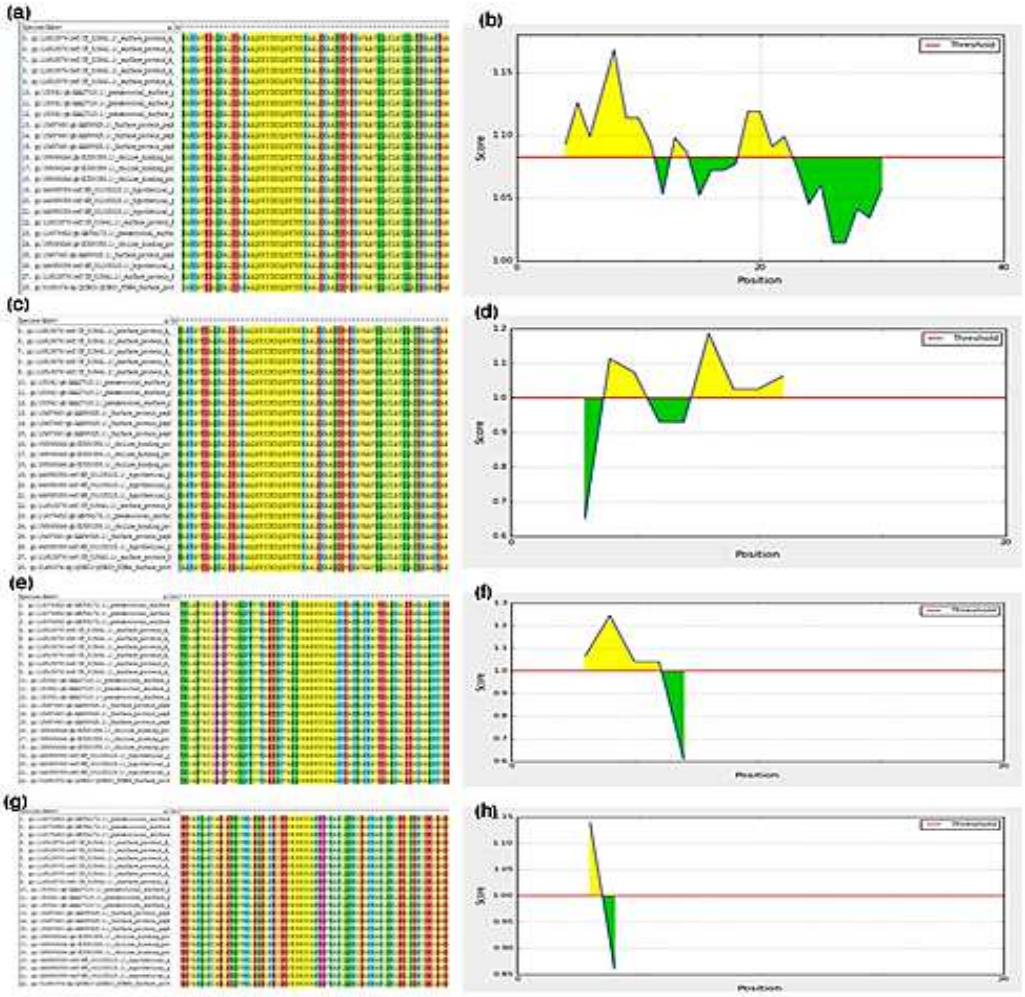


Fig. 2: Multiple Sequence Alignments (MSA) showing conserved region performed by MEGA 7.0. (a) ILTSLASVAILGAGFVASQPTVVRAEESPVASQ; (c) AQKKYEDDQKKTEE; (e) SKAEKDYDAA; and (g) SESEDYA. Kolaskar & Tongaonkar Antigenicity Results for (b) ILTSLASVAILGAGFVASQPTVVRAEESPVASQ and Emini surface accessibility prediction for (d) AQKKYEDDQKKTEE; (f) SKAEKDYDAA; (h) SESEDYA

Codon optimization of the selected epitope sequences

This study incorporated synthetic polynucleotide sequences optimized for heterologous expression of PspA in plant systems. Sequences were selected after screening multiple serotypes in GenBank using BLAST, prioritizing those most frequently shared across different serotypes and validated through the IEDB Analysis Resource for epitope prediction (Fig. 2).

Codon optimization was performed to ensure efficient translation of the transgene-encoded protein. The efficiency of translation can be estimated by calculating the Codon Adaptation Index

(CAI) for the gene of interest relative to the codon usage of the target organism. It is well established that synonymous mutations, alterations that do not change the encoded amino acid, can influence protein expression levels by up to 1000-fold and may also affect protein conformation, post-translational modifications (PTM), stability, and function [26].

Codon usage bias refers to the unequal frequency of synonymous codons in coding DNA. Although 64 codons exist (61 amino acid-encoding and 3 stop codons), only 20 amino acids are encoded, resulting in degeneracy in the genetic code whereby most amino acids are specified by more than one codon. Different organisms preferentially use certain synonymous codons over others at frequencies exceeding random expectation, a phenomenon that remains an active area of debate in molecular evolution. Organism-specific codon usage bias data are accessible through databases such as the HIVE-Codon Usage Table, which compiles genomic codon usage information from GenBank and RefSeq entries (Fig. 3).

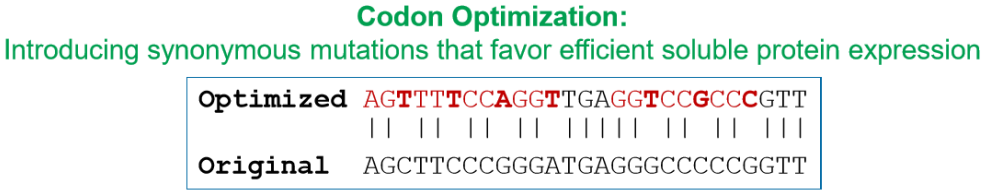


Fig. 3: Codon optimization for heterologous expression of PspA

Preparation of constructs

Vector Alpha zein fertig

The Vector Alpha zein fertig construct was designed to carry the alpha zein gene, into which the S. pneumoniae epitope 1 sequence was inserted as a fusion. To facilitate detection and localization, the alpha zein construct (construct alpha) incorporates both a FLAG-tag and a His-tag. The FLAG-tag consists of the sequence motif DYKDDDDK (D = aspartic acid, Y = tyrosine, K = lysine) and functions as an artificial antigenic marker (Fig. 4). Construct alpha is approximately twice the size of construct gamma, at around 1000 bp or 36 kDa. Both constructs were cloned into a binary vector containing an E. coli origin of replication and an ampicillin resistance gene. Specifically, ColE1 serves as the replication origin for E. coli, while RK2 functions as the origin for A. tumefaciens. The AmpR gene confers resistance to ampicillin in E. coli and carbenicillin in A. tumefaciens, and the nptII gene provides kanamycin resistance (Fig. 4).

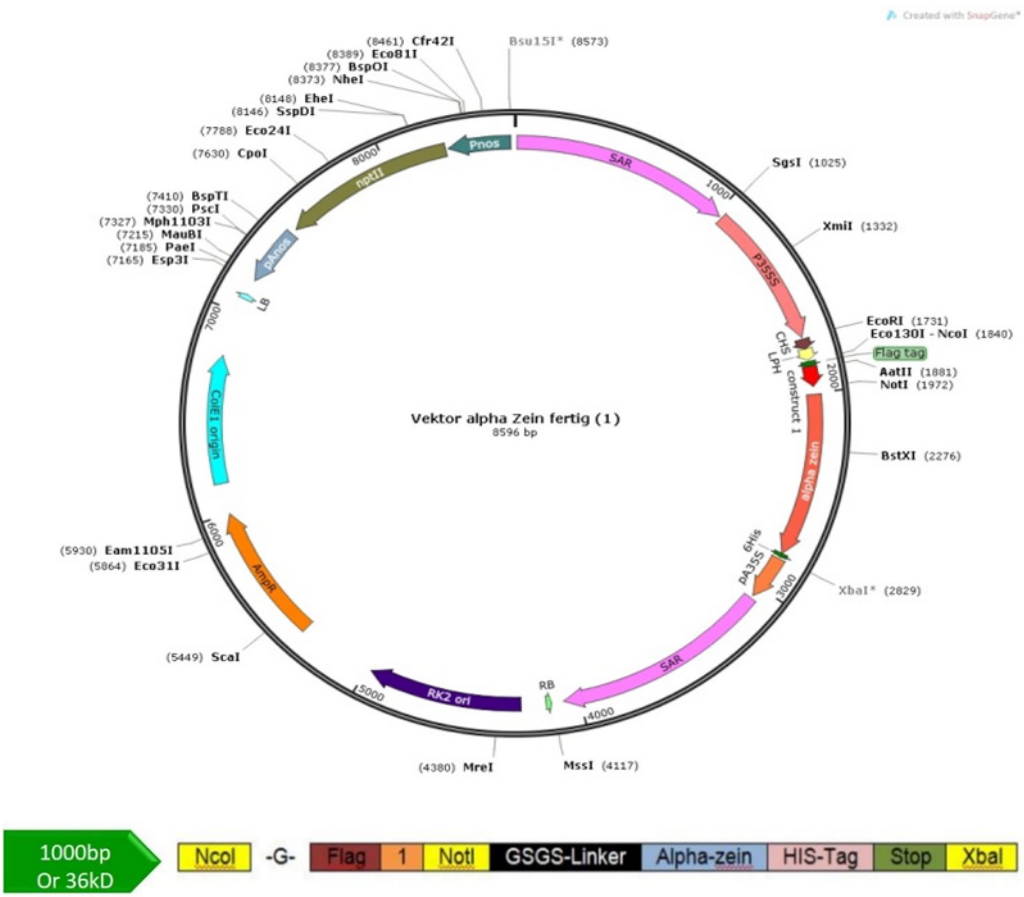


Fig. 4: This is an image of the final vector with alpha zein which is transferred into *A. tumefaciens* at the end Ncol, Notl and Xbal are the restriction sites. Construct 1 is the first sequence of *S. pneumoniae*.

Vector gamma zein fertig

The selected *S. pneumoniae* epitope sequences 2, 3, and 4 were fused to the N-terminal 90 amino acids of the 27 kDa gamma zein protein and cloned into the gamma zein fertig plasmid vector. Unlike construct alpha, the gamma zein construct (construct gamma) carries only a His-tag for detection purposes (Fig. 5). Construct gamma is approximately 500 bp in size, corresponding to a protein of around 18 kDa. All additional features of this construct are illustrated in Figure 5.

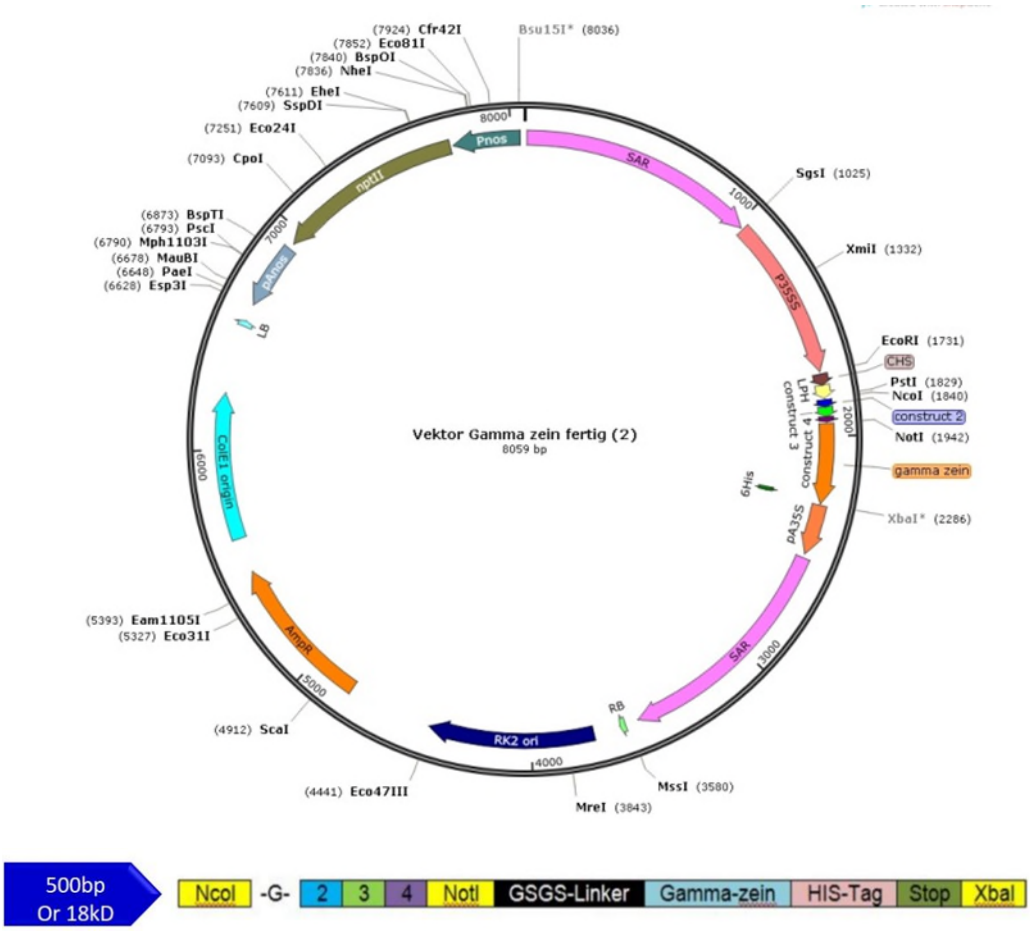


Fig. 5: This is an image of the vector with gamma zein. It is quite similar to the vector alpha zein. The differences are: (i) instead of alpha zein there is gamma zein (ii) there are 3 sequences of *S. pneumoniae* and (iii) there is only the his tag available for the localization and determination of the proteins.

The two constructs differ in size: construct gamma is approximately 500 bp, encoding an 18 kDa protein, while construct alpha is roughly double that size at 1000 bp, corresponding to 36 kDa. Both constructs were inserted into a binary vector harboring an *E. coli* replication origin and an ampicillin resistance gene. DNA concentrations were measured using a NanoDrop spectrophotometer, after which the vectors were subjected to double digestion with NcoI and XbaI. The digestion products were resolved on an agarose gel by electrophoresis at 70 V for 40 minutes.

Target-specific primers design

Primer-BLAST was used as it allows users to design new target-specific primers in one step as well as to check the specificity of pre-existing primers.

The selected Primers:

Finally the primers that have been selected for those of the target gene e.g. Alpha zein, and Gamma zein are given bellow-

Alpha zein:

Forward primer	ATCCTGGGTGCTGGTTTTGT
Reverse primer	TTGCTGTTGCAATTGTGCGA

Gamma zein:

Forward primer	AGAAAGACTACGACGCTGCC
Reverse primer	GAGAGGGATGCGGTTGTTGA

Agrobacterium-mediated genetic transformation

In this phase of the investigation, a series of experiments was performed to determine an appropriate protocol for Agrobacterium-mediated genetic transformation, utilizing two constructs, alpha zein and gamma zein, alongside the selectable marker gene nptII (Neomycin phosphotransferase). All vectors were constructed to include four selected B-cell epitope predicted peptide sequences derived from the Pneumococcal surface protein antigen (PspA). For this purpose, genetically engineered Agrobacterium strain LBA4404, harboring two distinct binary vector plasmids, namely alpha zein fertig and gamma zein Fertig, was employed in the transformation assays. Transformation experiments were carried out using various explants, including immature leaflets, cotyledonary nodes, hypocotyls, and single cotyledons of L. esculentum. During the regeneration experiments, it was observed that the regenerative capacity of immature leaf, cotyledonary leaf, and hypocotyl explants was comparatively higher than that of other explant types, such as the cotyledonary node in tomato. The outcomes of these experiments are presented below under separate headings.

PCR Confirmation of Alpha Zein and Gamma Zein Construct Integration into *Agrobacterium tumefaciens* LBA4404

Agrobacterium tumefaciens strain LBA4404 carrying the alpha and gamma zein constructions was used to extract genomic DNA, which was then amplified using construct-specific primers in order to confirm the successful integration of the target constructs. To ensure reproducibility, each primer pair included two separate replicates. The forward primer 5'-ATCCTGGGTGCTGGTTTTGT-3' and reverse primer 5'-TTGCTGTTGCAATTGTGCGA-3' were utilized for the alpha zein construct (lanes 3–4, Fig. 6a). The forward primer 5'-

AGAAAGACTACGACGCTGCC-3' and reverse primer 5'-GAGAGGGATGCGGTTGTTGA-3' were utilized for the gamma zein construct (lanes 8–9, Fig. 6a). The 1 kb DNA ladder served as a size reference in lane 1. All primer pairs formed distinct bands of the anticipated sizes after agarose gel electrophoresis, indicating that both constructs were successfully present in the corresponding Agrobacterium LBA4404 strains utilized for the next plant transformation studies (Fig. 6a).

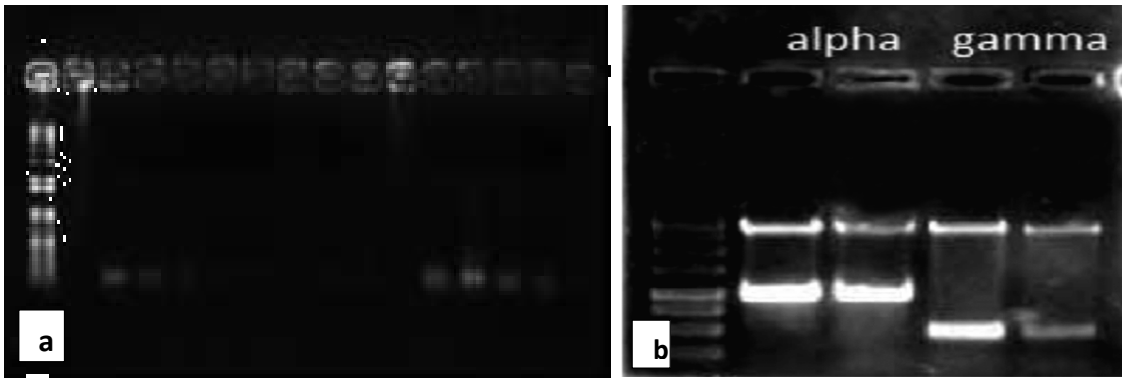


Fig. 6: (a) PCR confirmation of the presence of the target gene in *Agrobacterium* LBA4404 using specific primers: Lane 3-7 for alpha zein; lane 8 to 10 for gamma zein; (b) The agarose gel after the electrophoresis of the digestion mix. Lane 1 is the ladder; Lane 2 and 3 are construct alpha, and Lane 4 and 5 are construct gamma.

Confirmation of the presence of target gene into *Agrobacterium* LBA4404 by restriction digestion

Figure 6b displays the gel following electrophoresis. The first two lanes correspond to the digested alpha zein vectors, while the subsequent lanes represent the gamma zein vectors. The upper bands, which are of identical size, correspond to the vector backbones, and the lower bands represent the inserts. The alpha zein insert is approximately twice the size of the gamma zein insert. Restriction digestion analysis subsequently confirmed that *Agrobacterium* LBA4404 harbored the genes of interest.

Selection of different transformed shoots of *L. esculentum* using kanamycin

A kanamycin concentration of 50 mg/l was initially applied as the first selection pressure, after which the kanamycin concentration was progressively increased in steps of 100 mg/l, 150 mg/l, 200 mg/l, 250 mg/l, and 300 mg/l. Shoots that remained green and healthy and survived on medium containing the optimal selection pressure (100 mg/l) for 30 days were selected as putatively

transformed shoots. The various stages of regeneration and selection of transformed shoots, along with gene integration for alpha zein and gamma zein in *L. esculentum*, are illustrated in Fig. 7a. In all cases, the survival of green, healthy plants following kanamycin treatment confirmed successful transformation. The outcomes of the transformation experiment are presented in Table 1.

Following co-cultivation, the explants were transferred to selection medium after 10–15 days. Among the constructs tested, alpha zein yielded approximately 220 shoots recovered from about 530 infected explants on the final selection medium [27]. Thus, the recovery frequency of putatively transformed shoots from cotyledon explants was approximately 41.50%. For gamma zein, the recovery frequency of putatively transformed shoots was approximately 51.02% (Table 1).

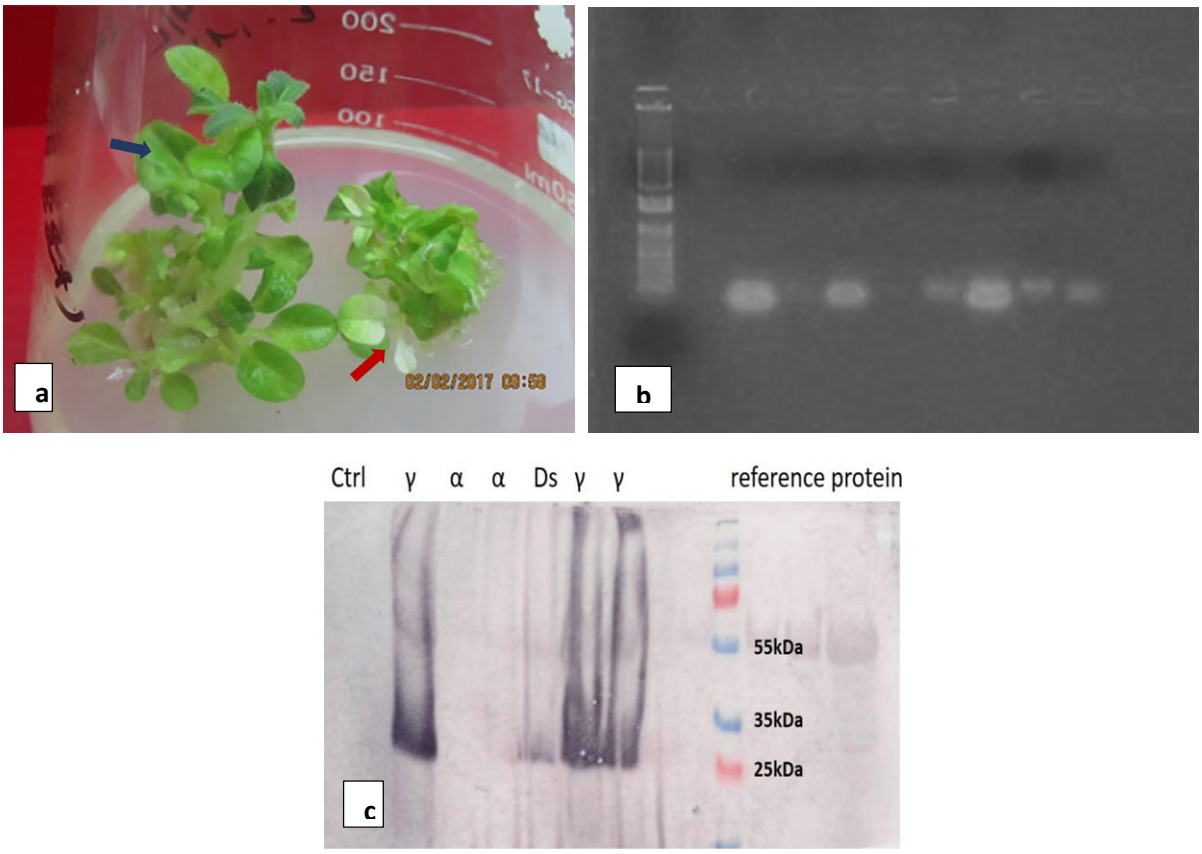


Fig. 7: (a) Survived regenerated transformed shoots of *L. esculentum* on medium containing 100 mg/l kanamycin (blue arrow-transformed part, red arrow-albino); (b) PCR analysis for the amplification of alpha zein, and gamma zein gene in *L. esculentum.* In this picture lane 1=ladder 1kb. Lane 3 to 8 for alpha zein; and 10 for gamma zein gene; (c) The recombinant proteins were detected using an anti-His-tag antibody (γ = gamma zein fusion, and α = alpha zein fusion. Ctrl=control).

Table-1: Effect of kanamycin on selection of infected shoots of *L. esculentum* transformed with the constructs alpha zein, and gamma zein.

Sl. No	Constructs	No. of explants infected	No. of survived shoots in regeneration media with 50 mg/l kanamycin	% of survived shoots
1.	Alpha zein	530	220	41.50
2.	Gamma zein	490	250	51.02

PCR analysis for the amplification of alpha zein, and gamma zein in *L. esculentum*

In *L. esculentum*, genomic DNA extracted from 8 putatively transformed rooted plantlets was subjected to PCR analysis to amplify the alpha zein and gamma zein genes. For both genes, amplification was observed in nearly all plants, which produced bands identical to that of the positive control (Fig. 7b).

Immunoblot analysis of T_0 plants

Tomato leaves were transformed using construct alpha and construct gamma. Plant samples were collected from the transformed leaves and immediately shock-frozen in liquid nitrogen to prevent protein degradation. These samples were then prepared for SDS-PAGE, followed by western blotting.

The membrane was incubated with antibodies directed against the His6-tag. A stronger band was observed for construct gamma compared to alpha zein. Unexpectedly, the molecular mass of the gamma fusion protein was substantially greater than anticipated (Fig. 7c).

Immunization testing of Mice

Following six weekly immunizations (weeks 0-5) with transgenic plant tissues, all experimental mice (groups 1-3) and controls were challenged with live *S. pneumoniae* serotype 7F and monitored for 30 days. Significant differences in survival emerged between immunized and control

groups during the first trial. The control group exhibited 50% mortality (6/12 mice) within 8-10 days post-challenge. In contrast, immunized mice showed markedly improved survival:

Alpha zein group: 83.3% survival (10/12 mice), with only two fatalities occurring by day 16

Gamma zein group: 75% survival (9/12 mice), with three deaths occurring by day 16

All surviving mice remained healthy throughout the 30-day observation period, demonstrating complete protection against the lethal challenge (Table 2).

Comparative study of mice challenge experiment

Similar results were obtained in the 2nd trial experiment. In both cases the survival rate for control is 50%, and in case of alpha zein there is no significant differences in survival rate between 1st and 2nd trial. In case of 2nd trial, using gamma zein. 50% survival rate were found in both cases, indicating that these results were significant (Fig. 8a).

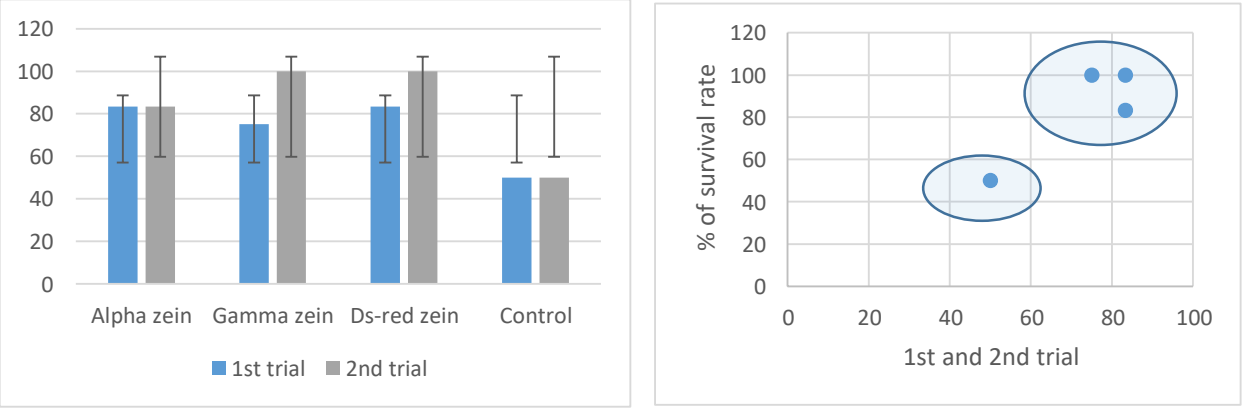


Fig. 8: (a) Comparative study after Mice challenge experiment. The results are expressed as mean ± standard deviations; (b) Relationship between control and immunized mice survival rate.

Two cluster groups in this scatterplot showed significant variation between control and immunized mice. The distance between these two cluster indicate that significant variation is present between control and immunized mice on survival rate (Fig. 8b).

All the tests were performed with SPSS of its version 22.0. The results of statistical analyses of mice challenge test was given bellow:

Table-2: Comparison of 1st and 2nd trials of mice challenge test.

Group Statistics					
	trial	N	Mean	Std. Deviation	Std. Error Mean
Survival	Trial 1	4	74.1650	17.02243	8.51121
	Trial 2	4	83.9275	23.60016	11.80008

Independent Samples Test										
		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Survival	Equal variances assumed	0.355	0.573	-.671	6	0.527	-9.76250	14.54932	-45.36340	25.83840
	Equal variances not assumed			-.671	5.457	0.530	-9.76250	14.54932	-46.24072	26.71572

In order to test the equality of average survival number from two trials (1st and 2nd), t-test has been performed. Test results shows that the means are not significantly different ($p>0.05$). That means, the survival rate in both the trials are almost similar.

There is no significant variation in between 1st and 2nd trial of mice challenge experiment.

Oneway

ANOVA					
Survival					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2275.928	3	758.643	6.671	0.049
Within Groups	454.877	4	113.719		
Total	2730.805	7			

To test the equality of survival rate in control group and three other constructs namely Alpha, and Gamma, Analysis of Variance (ANOVA) has been done. It can be seen from the test results that average survival rates in the three constructs are different from the control group ($p<0.05$).

Post Hoc Tests

Homogeneous Subsets

Survival			
Duncan[a]			
Inserts	N	Subset for alpha = 0.05	
		1	2
Control	2x12	50.0000	
Alpha Zein	2x12		87.0200
Gamma Zein	2x12		87.5000
Sig.		1.000	0.689
Means for groups in homogeneous subsets are displayed.			
a. Uses Harmonic Mean Sample Size = 2.000.			

For further understanding of the ranks of the average number of survival rates in the three groups, Duncan Multiple Ranked Test (DMRT) has been applied. It can be noticed from the findings that all the constructs are in one subset, and they are far higher than control set which belongs to another group at 5% level of significance.

Discussion

Notwithstanding the availability of antibiotics and licensed pneumococcal vaccines, *S. pneumoniae* continues to impose a substantial global disease burden. Conventional vaccine platforms are constrained by high production costs, cold-chain dependency, and complex distribution logistics, underscoring the need for innovative alternatives.

Bioinformatics analyses revealed that the cluster epitopes would be able to stimulate an immune response in mice. It was determined that pneumococcal surface protein antigen (PspA) is in fact a highly conserved protein, and as such highly immunogenic peptides of PspA can serve as good vaccine candidates. In this investigation, after necessary sequence analyses using Gene bank (NCBI), protein analysis (IEDB), Protein Data bank (PDB), and different software use like Mega,

SnapGene viewer facilitated to hand-picked the epitope sequences of PspA. Thus, the immune-informatics analysis has predicted some multi-epitope clusters [28, 29, 30].

In the present study, the leaf discs of *in vitro* grown tomato plants were used for co-cultivation with *Agrobacterium* and the transformed callus tissue was obtained by applying cefatoxime, kanamycin, and carbenicilin which prevents the growth of other bacterial cells, prevent excessive growth of *Agrobacterium* and allows only the kanamycin resistant ones to grow [21].

In this study, transgenic tomato and tobacco plants expressing the PspA virulent factor of *S. pneumoniae* specific antigen Alpha, and Gamma were developed fused to the zein protein. To achieve high expression and accumulation of these fusion proteins in the selected plant, a highly active promoter was used to express the synthetic vaccine sequences. Synthetic vaccine sequences were codon-optimized for expression in plants, as it has been reported that such an approach results in increased protein expression [22]. The transformants developed through this study were characterized through molecular analysis, like PCR and immunoblot analysis to confirm the introgression of foreign genes in nucleotide and protein levels.

As an integral part of *Agrobacterium*-mediated genetic transformation, experiments were carried out to establish an efficient *in vitro* regeneration protocol using plants *L. esculentum*, which are commonly cultivated in Bangladesh.

The constructs used in this investigation contained *npt*II gene within its T-DNA region and this gene confers kanamycin resistance to the transformed cells. Therefore, for the selection of transformed tissue kanamycin was applied to the regeneration medium for the selection of transformants. To determine the level of selection agents (kanamycin) in medium, different concentrations of kanamycin were tested. In many reports, kanamycin was applied immediately after co-cultivation for the selection of transgenic shoots/cells [23, 24]. However, in the present study it was observed that even in the presence of a lower concentration of kanamycin, co-cultivated explants failed to regenerate. After selection it was observed that all control shoots died in the selection medium with 50 mg/l kanamycin. Non-transformed shoots started to become albino in presence of 50 mg/l kanamycin.

In alpha zein along with His tag also FLAG-tag inserted for the detection purpose. Surprisingly, the mass of the gamma fusion protein was much higher than expected. One reason could be the formation of protein dimers. The mass of a dimer would correspond to the result [25].

In this study tomato transformed leaves were orally fed to 36 mice divided into three groups. The groups were divided by alpha, gamma, and one control (Non-transformed). In both of the 1st and 2nd trials, among 36 orally immunized mice, 80.55% mice remain alive after challenge with *S. pneumoniae* of serotype 7F in case of 1st trial and 95.23 in case of 2nd trial. In case of control (Mice feeding non-transformed leaves), the rate is 50% in both trials. Therefore, mice that received 1gm of transgenic leaves over the course developed a better immune response to PspA than those fed in non-transformed leaves.

After statistical analyses using SPSS version 22.0, it has also been proven that there is no significant difference between the first and second trials of the mice immunization test. In order to test the equality of average survival number from two trials (1st and 2nd), t-test has been performed. Test results show that the means are not significantly different ($p>0.05$). That means, the survival rate in both trials are almost similar. So, there is no significant variation between 1st and 2nd trials of mice challenge experiment.

To test the equality of survival rate in control group and three other constructs namely Alpha, and Gamma, Analysis of Variance (ANOVA) has been done. It can be seen from the test results that average survival rates in the three constructs are different from the control group ($p<0.05$).

After applying Duncan Multiple Ranked Test (DMRT), it can be noticed from the findings that all the constructs are in one subset, and they are far higher than the control set which belongs to another group at 5% level of significance.

Although the present study successfully demonstrated the development of transgenic plants expressing conserved PspA epitopes and showed significant protective efficacy in the murine challenge model, detailed immunological analyses such as antibody titer determination, ELISA, mucosal IgA response, cytokine profiling, and cellular immune response evaluation were not included in this investigation. The current work represents the outcome of more than seven years of extensive experimental research involving epitope prediction, construct preparation, plant transformation, molecular confirmation, and animal challenge studies. Comprehensive immunological characterization requires additional long-term, labor-intensive, and resource-

demanding experiments, which are planned as the next phase of this research. Future investigations will therefore focus on detailed immunogenicity and immune mechanism analyses of the developed edible vaccine candidate.

Conclusion

From the foregoing discussion, it may be concluded that the preparation of the constructs, the transformation into *A. tumefaciens* and the expression of gamma zein, and alpha zein as intact fusion proteins with Pneumococcus antigen in plants were successful. These immunogenic hot spot within PspA has the potential to serve as an attractive candidate for the development of a novel pneumococcal vaccine.

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Declarations

The submitting research article "Development of an Edible Vaccine Using Conserved PspA Epitopes Against Pneumococcal Infection in a Murine Model" for publication in your journal of repute, is a unique article, and to the best of our knowledge, this is among the few studies reporting the development of a plant-based edible vaccine using conserved PspA epitopes of *S. pneumoniae* combined with Agrobacterium-mediated transformation and murine challenge analysis.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board (IRB) of the Dean, Faculty of Biological Sciences, University of Dhaka, Dhaka, Bangladesh (Reference No. 363/Biol. Scs.2025-2026). This investigation was carried out in accordance with the ARRIVE guidelines and institutional animal ethics approval.

Consent to publication

Not applicable.

Data availability statement

All data generated or analysed during this study are included in this published article. Additional raw data are available from the corresponding authors upon reasonable request.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Author's Contributions:

SA designed the study and performed the experimental work. SA, TAB, BG helped in performing plant transformation experiments. SA and MNU contributed in statistical analysis. SK and CRA helped to provide logistical and laboratory support. ES, JN and CRA participated in supervising and reviewing the draft and thoroughly checked and revised the manuscript for necessary changes in format. CRA also acted for all correspondence. All authors read and approved the final version of the manuscript.

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