Development of a stable vaccine formulation which is effective, safe and stable is very critical especially when purified proteins are used as vaccine antigens. The need for an effective vaccine against Human Papilloma Virus infection which could elicit a very sustainable immune response in both systemic & mucosal system and possibly a needle free immunization is well justified by the given situation of the disease condition. The current study used a purified HPV protein as vaccine antigen and different formulations using Aluminium adjuvants and Niosomes were prepared and studied the immune response with two different routes of administration viz., subcutaneous and intranasal.

**Characterization of the antigen:** The characterization of HPV L1 protein was performed using SDS PAGE to study the stability of the protein under different physico-chemical conditions. The SDS PAGE profile in native and denatured condition during freeze thaw conditions demonstrated that the protein stability profile.

The protein was exposed to different pH and temperature conditions and studied the stability of the protein under those conditions. The stability of the protein in the presence of a stabilizer such as sugar additive in the extreme conditions also was studied. The conclusions of the study under native & denaturing conditions are, i) HPV protein is stable at pH conditions, pH 5.7 to 6.5 ii) Degradation is much lesser and slower at 4°C compared to room temperature (25°C) and stress conditions (37°C) iii) Inclusion of sugar additives (sucrose, sorbitol and trehalose), inclusion of glycerol and polyethylene glycol had no beneficial effect on the stability, when stored at extreme pH and temperature conditions. Maximum instability was seen at pH 4.0. The degradation is much lesser and slower at 4˚C as compared to room temperature and 37˚ C. The inclusion of EDTA (metal chelator) had beneficial effect on the stability of protein as it prevents the oxidation of the protein. It is also recommended to use pharmaceutical grade raw materials (like Indian Pharmacopeia, British Pharmacopeia, United State Pharmacopeia and Japanese Pharmacopeia) to avoid metal impurities from raw materials.

The stability under freeze thaw condition: The conclusions of the study are; i) -20°C stored proteins was more stable than protein stored 4°C. ii) At all Temperatures, HPV protein was more stable and at pH 5.9 compared to pH 7.0 and pH 7.5; iii) Gel analysis indicates that, although no degradation was observed, decrease in intensity of
HPV protein seen at 4°C. Only characterized and qualified antigens were used in the formulation studies that were selected for *in-vivo* potency studies.

The aim of the research was to study about the immune response of HPV L1 antigen formulated in Aluminium adjuvants and compare the performance of Niosome formulations. The antibody response from each immunized group was studied using ELISA from serum and vaginal fluid samples collected from the animals at different intervals after immunization, with and without booster doses. The response was evaluated based on the optical density (OD) at 450 nm and 490 nm of test samples (serum and vaginal fluid respectively) against a control sample collected from animal immunized with a placebo.

**Aluminium Adjuvants formulations:** The initial experiments with Aluminium adjuvants demonstrated that Aluminium phosphate adjuvant was more effective than Aluminium Hydroxide, in eliciting an acceptable level of antibody response in the serum samples. Although the response was lower with a single immunization dose at day 21, the response improved significantly with the booster doses. It was also observed that Aluminium adjuvant do not induce any mucosal antibodies even after two booster doses.

However, the Aluminium Hydroxide could not elicit any significant level of antibody response even after two booster doses demonstrating that Aluminium Hydroxide was not the suitable adjuvant for this protein. This could be due to the difference in the Isoelectric point of the Aluminium Hydroxide against the protein which impacts the antigen adsorption due to electrostatic attraction. It was also demonstrated in the protein adsorption study that the protein could not be adsorbed to an acceptable level as compared to Aluminium phosphate which is above 90% adsorption.

It was clearly demonstrated that neither Aluminium phosphate nor Aluminium Hydroxide could elicit any significant level of mucosal immune response as tested in vaginal fluid. Though the serum antibody level improved over booster doses, no measurable response was demonstrated in vaginal fluid.

**Niosome formulations:** Non-ionic surfactant vesicles (Niosomes) have demonstrated their ability to function as adjuvants following parenteral administration with a number of different antigens and peptides for eg, synthetic measles peptide (Brewer, Roberts *et al.*
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1996) and HSV-1 (Hassan, Brewer et al. 1996). Also NISV have been reported to possess extremely low toxicity (Brewer, Roberts et al. 1994).

**Subcutaneous Route of Administration:** The experiments using Niosomes containing different concentrations of HPV L1 antigen provided very significant information about their ability to induce both systemic and mucosal immune response. The Niosomes containing 0.5 mg/mL or 0.75 mg/mL of HPV protein demonstrated equal or better immune response as compared to Aluminium phosphate, when immunized in subcutaneous route. The study comparing the response of Niosome formulation containing 0.5 mg/mL antigen and 0.75 mg/mL protein demonstrated that increase in the payload did not have any beneficial effect on the *in-vivo* response. However, in contrast to Aluminium phosphate, these Niosome formulations when injected in subcutaneous route (similar to Aluminium adjuvant) could elicit a meagre level of immune response in the mucosal surface as tested in vaginal fluid. This mucosal immune response obtained at the 60 day sample was only comparable with the response obtained in serum using Aluminium phosphate adjuvant at Day 20.

The experiment with Niosomes containing CTB, bacteria derived adjuvant, when injected subcutaneously, demonstrated a similar response in the serum at three different time points. However, the mucosal immune response was significantly higher (*P < 0.01*) when compared to the simple Niosome preparations and Aluminium adjuvants containing only the antigen.

**Intranasal Route of Administration:** Induction of antibodies in mucosal secretions (sIgA) is not only dependent on the particular secretion examined, but also on the site of mucosal immunization, i.e., airway, oral, rectal or genital. Hence in this study, we were interested in inducing anti HPV antibodies mainly in the vaginal secretions, as the cervix is the prevalent site of high-risk types HPV infections. Secretory immunity results from activated B cells arriving from inductive mucosa associated lymphoid tissue (Brandtzaeg *et al.*, 2007, Neutra *et al.*, 2006). Such mucosa-associated inductive sites not only differ in their location, but also in their ability to efficiently mount specific immune response. The latter being more or less regionalized pending on whether activated B cells may only home back to the mucosa they originated from or mucosa situated at more distant sites (Neutra *et al.*, 2006). For instance the sparse lymphoid aggregates associated to the
cervico-vaginal mucosa are less prone to mount efficient immune responses, while nasal associated lymphoid tissues are efficient at inducing antibody responses in the respiratory secretions as well as in the distant genital mucosa (Mestecky et al., 1997). In addition, such properties may differ depending on the antigen and the adjuvant used.

Although sIgA are particularly well suited to protect mucosa through both immune exclusion and intracellular neutralization (Brandtzaeg et al., 2007), serum derived HPV–L1 specific antibodies induced by intramuscular HPV vaccination, appear to be sufficient to provide close to 100% protection against persistent HPV infection and associated pre-neoplastic lesions. Moreover, additional induction of sIgA may still be of benefit for long term protection in ovulating women.

To demonstrate this, intranasal immunization was done using Niosome containing only 0.5 mg/mL antigen and formulation containing antigen with CTB. Both the formulations did not elicit any response in serum at day 20 and hence an additional booster was given and the response was estimated at day 42 & day 60.

The Niosome formulation containing 0.5 mg/ml antigen elicited a comparable immune response at day 42 and day 60 similar to subcutaneous. The average OD values were more than 2.0 at day 42 and day 60 in both serum and vaginal samples.

The Niosome formulation containing 0.5 mg/ml antigen with CTB also elicited a similar immune response at day 42 and day 60 like the simple Niosome formulation when given intranasal. The average OD values were more than 2.0 at day 42 and day 60 in both serum and vaginal samples, and the response between these two formulations were not significantly different (p>0.05).

This formulation with three booster doses, elicited a better immune response in both serum and mucosal surfaces. Also the mucosal response was comparable with the response obtained with the same formulation given subcutaneously. The Niosome formulation with CTB did not show adequate immune response without booster. This demonstrates a possible early clearance of these Niosome formulated material from the nasal cavity.

Ideally, pay load of parenteral vaccination requires lower pay load as compared to mucosal routes viz. oral, nasal, anal etc. Based on the immunological results observed with Niosome formulations with and without CTB when administered in intranasal route.
with an additional booster, elicited a significant immune response in serum and vaginal fluid even though the amount is little lesser than obtained with subcutaneous route. In this case it is demonstrated that formulations administered by mucosal route (nasal route) required lower pay load to raise a response as compared to formulations administered by parenteral route. This could be due to strong bioadhesion in the nasal cavity and prolonged antigen presentation to APCs. Generally, bioadhesion can be promoted by a positive zeta potential value. It is to be noted that all the niosome formulations had a zeta potential values above +20. Such bioadhesive properties are thought to be the result of electrostatic interaction between the positively charged amino groups on the positively charged formulations and negatively charged sialic acid groups in the mucin network in nasal cavity. However, the need for an additional booster dose confirmed the fact that few critical factors such as i) difference in pay load as compared with subcutaneous and ii) possible early nasal clearance of the Niosomes need to be studied further in detail.

The experiment conducted to study the immune response without booster doses proved that, booster doses are required for an effective response. Both serum & vaginal fluid did not elicit an acceptable level of response when tested at day 60, demonstrating the need for booster.

The studies conducted to determine the stability of different formulations demonstrated that both Aluminium phosphate formulation and Niosome formulation was proved to be stable till day 180 when stored at 2-8°C. All these formulations proved to be stable at 25°C up to 60 days, although the day 60 response was lower than the cold storage condition. However, the study needs to be continued further to determine the stability profile of these formulations.

Hence a significant systemic and mucosal immune response against HPV 16 L1 antigen could be elicited with Niosome formulations with and without CTB when immunized through subcutaneous and intranasal route, and the study further can focus on the following aspects to make a robust vaccine formulation. They are;

i) Dose standardization study for single dose immunization.

ii) Antibody Neutralization efficiency

iii) The deposition and subsequent clearance studies of the Niosome formulations given through intranasal route (gamma scintigraphy); Nasal clearance Rate study.