

Vaccination of Goats with Fresh Extract from *Sarcoptes scabiei* Confers Partial Protective Immunity

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ABSTRAK

TARIGAN, S. 2006. Vaksinasi kambing dengan ekstrak segar *Sarcoptes scabiei* menghasilkan kekebalan parsial. *JITV* 11(2): 144-150.

Telah diketahui bahwa kekebalan yang melindungi terbentuk saat *Sarcoptes scabiei* menginfestasi hewan. Akan tetapi pada penelitian terdahulu usaha untuk menginduksi kekebalan dengan memvaksin kambing dengan fraksi protein tungau baik yang larut maupun yang 'nirlarut' tidak berhasil. Degradasi atau denaturasi protektif antigen saat preparasi vaksin merupakan salah satu kemungkinan kegagalan tersebut. Pada penelitian ini, protein tungau yang dipakai disiapkan dengan cepat untuk menghindari terjadinya degradasi atau denaturasi protein. Sebanyak 150 mg tungau yang baru saja diisolasi dengan cepat dihomogenisasi, disentrifus dan dipisahkan menjadi fraksi supernatan dan pelet. Sebanyak 28 kambing dibagi menjadi 4 kelompok sama banyak. Kelompok 1 divaksin dengan keseluruhan homogenat, kelompok 2 dengan supernatan, kelompok 3 dengan pelet, dan kelompok 4 dengan PBS (kontrol). Vaksinasi dilakukan sebanyak 3 kali, dengan interval 3 minggu, menggunakan Quil A sebagai adjuvan, dan setiap vaksinasi menggunakan homogenat yang segar. Satu minggu setelah vaksinasi terakhir, semua kambing ditantang dengan kira-kira 2000 tungau hidup. Keparahan lesi, yang diskor dari 0 (tanpa lesi) sampai 5 (>75% telinga yang terkena lesi) ditentukan satu hari, dua hari, lalu setiap minggu pasca tantang. Kerokan kulit untuk menentukan densitas tungau diambil delapan minggu pasca tantang. Penantangan dengan tungau menyebabkan terbentuknya lesi kulit pada semua hewan. Tidak ada perbedaan yang nyata antara kelompok hewan yang divaksin dengan yang tidak divaksin dalam hal keparahan lesi. Akan tetapi, densitas tungau pada kelompok hewan yang divaksin secara nyata ($P = 0,015$) lebih rendah dibandingkan dengan hewan yang tidak divaksin. Penelitian ini memberi indikasi bahwa protektif antigen *S. scabiei* sangat rentan terhadap degradasi atau denaturasi, dan terdapat dalam konsentrasi atau antigenisitas yang sangat rendah. Oleh karena itu metoda konvensional, fraksinasi keseluruhan protein tungau dan pengujian setiap fraksi dengan uji vaksinasi, kelihatannya tidak cocok untuk *S. scabiei*.

Kata Kunci: *Sarcoptes scabiei*, Vaksinasi, Homogenat Segar, Partial Immunity

ABSTRACT

TARIGAN, S. 2006. Vaccination of goats with fresh extract from *Sarcoptes scabiei* confers partial protective immunity. *JITV* 11(2): 144-150.

Protective immunity has been known to develop in animals infested with *Sarcoptes scabiei*. However, our previous attempt to induce protective immunity in goats by vaccination with fractions of soluble or insoluble mite proteins had been unsuccessful. Degradation or denaturation of protective antigens occurred during vaccine preparation was suggested as one possible cause of the failure. In this study, mite proteins that used to immunise animals were prepared rapidly in order to prevent protein degradation or denaturation. About 150 mg of freshly isolated mites were rapidly homogenised, centrifuged then separated into supernatant and pellet fractions. Twenty-eight goats were allocated equally into 4 groups. Group-1 goats were vaccinated with the whole mite homogenate supernatant, group 2 with the supernatant, group 3 with the pellet, and group 4 with PBS (unvaccinated control). Vaccination was conducted three times, with three-week intervals between vaccinations, using Quil A as adjuvant, and each vaccination using fresh mite homogenates. One week after the last vaccination, all animals were challenged with approximately 2000 live mites. The severity of lesions, scored from 0 (no lesions) to 5 (>75% infested auricle affected), were determined one day, two days, then every week after challenge. Mite challenge caused the development of skin lesions in all animals. No significant differences between vaccinated and unvaccinated animals were observed in regards to the severity of lesions. However, the mite densities in vaccinated animals were significantly lower ($P=0.015$) than those in unvaccinated control. This study indicates that the protective antigens of *S. scabiei* are liable to degradation or denaturation and exist in a very low concentration or have vary low antigenicity. This implies isolation of the protective antigens by the conventional approach, fractionation of the whole mite proteins and testing each fractions in vaccination trials, is seemingly inappropriate for *S. scabiei*.

Key Words: *Sarcoptes scabiei*, Vaccination, Fresh Homogenate, Partial Immunity

INTRODUCTION

Scabies or sarcoptic mange, a contagious pruritic disease caused by *Sarcoptes scabiei*, is one of the most important disease in man and animals. It is estimated that over 300 million people are infected every year (ARLIAN, 1989). In animals, it is reported that more than 40 species of mites from 17 families and seven orders of mammals (ZÄHLER *et al.*, 1999). In domestic animals, pigs and goats seem to be the most susceptible, whereas in pet animals, the disease is most common in dogs. In wild animals, outbreaks of disease resulted in high mortality have been reported in gorillas (KALEMA-ZIKUSOKA *et al.*, 2002), ibex (LEON-VIZCAINO *et al.*, 1999), fox (FORCHHAMMER and ASFERG, 2000), and wombat (SKERRATT *et al.*, 1999).

Since the prevalence of the disease in man and animals is very high, the economic losses caused by the disease are enormous. Despite the huge economic losses, control measures that have been developed against this ectoparasite is limited. Until now, treating individual with clinical signs seems to be the most common method. This method is expensive and unpractical especially in animals. Topical scabicides is often ineffective, and very unpractical in animals especially when the number of animals to be treated is large. Injectable scabicides such as ivermectin is practical to use and usually effective for treatment clinical cases. However, this drug is expensive and intended for curative, not for preventive, purposes.

Although ivermectin is currently the drug of choice for scabies, dissatisfaction regarding the use of this antiparasite chemical is escalating. The consumers are now demanding that the animal products be free from all chemical residues, whether or not the residues are known to be harmful to their health or to the immediate environment (DONALD, 1994). Ample evidence has already been available to indicate that this chemical is unsafe to human health and the environment. Death in human associated with the ivermectin has been reported (BARKWELL and SHIELDS, 1997; BREDAL, 1997; COYNE and ADDISS, 1997). It has also been demonstrated that animals injected with ivermectin contained the chemical in their excrement that killed a variety of important dung colonising insects (WALL, 1992). Prolonged and improper use of pesticides, including ivermectin, usually resulted in the development of parasite resistance against the pesticide. The occurrence of resistance of *S. scabiei* against ivermectin has recently been reported (CURRIE *et al.*, 2004).

The increasing dissatisfaction against the use of anti parasite chemicals has provided the impetus to search for alternative means of parasite control. Vaccination seems to be an ideal alternative because it is practical, safe to the animals, consumers and the environment,

and it can be delivered with ease and potentially at low cost. However, developing antiparasite vaccines has proved to be much more difficult compared to antiviral or antibacterial vaccines. This is because interactions between the parasites and the host's immune system are much more complex. The principal component of parasitic vaccine, unlike that of bacterial or viral vaccines, usually consists a single protein that has the capacity to confer protective immunity. One of the difficulties lies on finding this protective protein which has to be searched among huge number of parasite proteins.

In our previous studies, both water-soluble and insoluble mite proteins were separated into several fractions, then the protective capacity of each fraction was evaluated in vaccination-challenge experiments. The experiments showed that none of the fraction has the capacity to induce protective immunity (TARIGAN, 2005; TARIGAN and HUNTLEY, 2005). It is unknown for sure why we failed to identify the protective proteins. It may be that the protective antigens are present in an extremely low concentration or the protective proteins had underwent inactivation due to denaturation or degradation during extraction or fractionation processes. One simple means to avoid protein denaturation and degradation is to rapidly extract and test the proteins. The purpose of this study, therefore, is to evaluate the protective capacity of freshly prepared mite proteins.

MATERIALS AND METHODS

Cultivation and collection of mites

A goat displaying clinical signs of sarcoptic mange was purchased from a nearby farm and transported to the laboratory. After confirmation by microscopic examination that *Sarcoptes scabiei* is the only parasitic inhabitant of the skin, the goat was used to infest healthy goats by housing them together in an isolated pen. This colony of many goats was maintained as source of mites for immunogen preparation and challenge of vaccinated animals. Goats exhibited severe dermatitis were euthanised with a mixture of ether and chloroform. The coat was clipped and shaved, and skin showing encrustation dermatitis was scraped deeply. The skin scraping was chopped into approximately 2-mm³ pieces, mixed thoroughly and kept at 4°C overnight. Mites were extracted using a previously described technique (TARIGAN, 1998). Briefly, the skin scrapings were placed at the edge of a Petri dish, and then a beam of light was directed to the centre of the Petri dish. Mites of all life stages crawling toward the light were collected by aspiration.

Preparation of soluble and insoluble mite proteins

Freshly isolated mites (approximately 150 mg) were washed in a solution of 1% SDS in PBS, then homogenised in 10 ml Kanamycin-PBS (PBS containing 100 mg kanamycin sulphate/ml). A 2-ml portion of the slurry was mixed with 2 ml kanamycin-PBS and referred to as fraction F_{TOT} . The remaining of the homogenate was centrifuged at $10\,000 \times g$ for 10 minutes then the supernatant was collected. A 3-ml portion of the supernatant was mixed with 1 ml kanamycin-PBS and referred to as fraction F_{SUP} . After being completely depleted from supernatant the pellet was resuspended in 4 ml kanamycin-PBS and this mixture was referred to fraction F_{PEL} . Each fraction was mixed (1:1) with a solution of Quil A (1 mg/ml in PBS) then injected immediately by intramuscular route into goats (1 ml per goats).

Animals and vaccination

Twenty-eight goats were randomly allocated into 4 groups (7 goats per group). Group 1 goats were vaccinated with the fraction F_{TOT} , Group 2 with the fraction F_{SUP} , Group 3 with the fraction F_{PEL} and Group 4 with Kanamycin PBS and adjuvant only. Vaccination was conducted three times with three week intervals between vaccination, and each vaccination utilised freshly prepared mite protein fractions.

Mite challenge and examination

One week after the last vaccination, all animals were challenged with approximately 2000 live mites on the left auricles using a procedure described previously (TARIGAN, 2003). Briefly, skin scraping was collected from a mangy goat, chopped into about 2-mm³ pieces, and mixed thoroughly. The number of mite per gram of skin scraping was determined by placing the skin scraping at the edge of a Petri dish. A beam of light was directed to the centre of the Petri dish, and mites migrating toward the light were counted under a microscope after 6 hours. A piece of cloth (6 x 4 cm²) was placed on the convex surface of a goat auricle, the bottom, left and right rims of the cloth were attached to the auricle by an adhesive tape. Skin scrapings containing approximately 2000 mites were inserted under the cloth through the top edge then the top edge was attached to the auricle.

After 48 hours, the cloth together with the skin scraping were removed and infestation was allowed to progress. Skin lesions caused by the mite challenge were photographed and their severity were assessed at 1 and 2 days, then at weekly intervals from one to eight weeks. The severity of lesions was determined arbitrarily by the following scores: score 0 if no lesion,

score 1 if <10% of the infested auricle was affected by the lesions, score 2 if 10-25%, score 3 if 25-50 %, score 4 if < 50-75%, score 5 if >75% or other parts of the body were affected. The effect of mite infestation on body condition was assessed from the changes in the body weight which were measured weekly before and after challenge.

ELISA

Antigen for the ELISA was prepared according to ARLIAN *et al.* (1994). The contaminated IgG in the mite proteins was depleted by passing the solution through a Protein-G-sepharose column (Amersham Pharmacia). Optimal concentration of immunoreagents were determined by checkerboard titrations using a positive reference serum (pooled sera from vaccinated goats) and a negative reference serum (pooled sera from prevaccinated goats). Peroxidase-conjugated-anti-goat IgG (Jekson Immuno Research Laboratories, West Grove, USA) was used as the secondary antibody, ABTS (Sigma chemicals) as chromogen and the reaction was measured at 405 nm. Antibody titre of a sample was expressed as the OD-ratio post-vaccinal or post challenge to pre-vaccinal serum.

Immunoblotting

Mites were homogenised in a Laemmli's –reducing-SDS-PAGE-sample buffer (65 mg/ml buffer). A 75 μ l portion of the supernatant was loaded into a single-sample-well-acrylamide plate (Protean II, BioRad). After electrophoresis, proteins from the acrylamide gel were transferred onto a nitrocellulose membrane (TARIGAN dan HUNTLEY, 2005). The membrane was cut into 5-mm-wide strips then soaked for 2 hours in a 0.1% solution of non-fat skim milk to blocked unoccupied sites in the membrane. The strips were reacted for 2 hours with goat sera at 1:1000 dilution. Alkaline-phosphatase-labelled-anti-goat-mouse-monoclonal antibody (Sigma) was used as detecting antibody and NBT/BCIP (Sigma) as substrates.

Histopathology and Mite Burden

At the end of challenge schedule (8 weeks after the initial mite infestation), the auricles were cut out at 5 cm from the tip after they had been previously infiltrated with a local anaesthetic, Lidocaine-HCl. After the biopsy, the incision site was sutured, smeared with an antiseptic solution (Betadine[®]) and sprayed with a screw-worm-and-wound-dressing spray (Gusanex[®]). The detached auricle was cut into halves, one half was fixed in buffered formol saline for histology and the other half was stored at -20EC to be used for mite count. The histological samples were

paraffin blocked, processed routinely and sections were stained with Haematoxylin and Eosin (H&E). The skin from the auricles that had been stored at -20EC were scraped and the scrapes were suspended in 20% KOH solution (scrapes obtained from 2 cm² skin were added to 1 ml KOH solution) for 30 minute. The scrape suspension was poured onto a 2-mm grid and the number of mites was counted under a light microscope.

Statistical analysis

The protective value of the immune response elicited by vaccination was based on the score of lesions, mite densities, titre of specific antibody measured by ELISA and changes in body weight. Differences in the score of lesions between group of goats were analysed using a non-parametric analysis, Kruskal-Wallis-one-way ANOVA (PETRIE and WATSON, 2001). Differences in the antibody titres, body weight changes and mite densities were analysed with a one-way ANOVA (PETRIE and WATSON, 2001). The data of mite densities which were expressed in number of mites/ cm² were first transformed into their logarithmic values before analysed with a one-way ANOVA (PETRIE and WATSON, 2001). When differences between groups were statistically significant (P<0.05), Tukey’s multiple comparison analysis were performed.

RESULTS

Examination of the animals one and two days after challenge revealed that infestation was established in all

animals. Some auricles became swollen with serocellular exudates oozed from the site of infestation, while others had numerous palpable small papules at the site of infestation. No differences were seen in the type or severity of lesions between group of goats. At one week, lesions developed into thick moist serocellular crusts or thin dry parakeratotic crusts with coats being fallen off at the site of infestation in all animals. In the following weeks, lesions progressed slowly. At four weeks, crusts of various thickness and alopecia were the most common lesions. In some animals, the crusts disappeared, leaving an area with alopecia but a healthy condition. At five weeks, however, the lesions seemingly reactivated as the area of encrustation dermatitis rapidly enlarged. Parakeratotic scales were formed in the alopecic skin that at previous weeks appeared to recover. At eight weeks, the severity of lesions varied between animals. In the majority of animals, however, about halve the surface of infested auricles were affected and no significant differences between vaccinated and unvaccinated, or among the vaccinated groups were observed (Figure 1).

The density of mites in the skin of infested auricles was presented in Figure 2. The mite densities were considerably low in all animals. Skin scraping collected even adjacent to the site of infestation contained no more than 5 mites/ cm². Although the severity of lesions were comparable in all group of animals, the density of mites in the unvaccinated control goats was significantly higher than those in the vaccinated group (P=0.015).

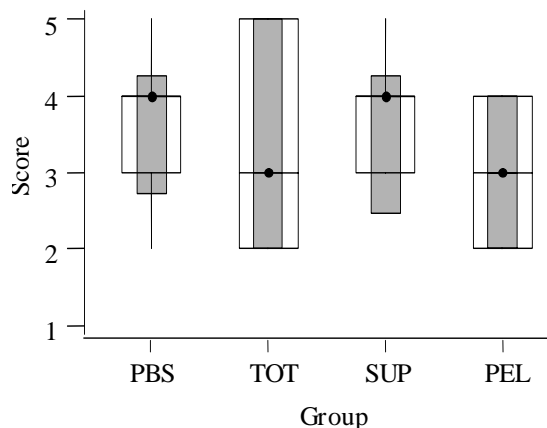


Figure 1. Box-and-whisker plot of score of lesions on the infested auricles. The lesions were score from 0 if no lesions to 5 if the more than 75% of the auricle affected by encrustation dermatitis. The solid boxes denote 95% confidential interval (CI) of the median, empty boxes denote internal quarter (IQ) range, the horizontal line with a dot in the middle denotes the median. Note that the severity of lesions between group of goats were not significantly different (P>0.05)

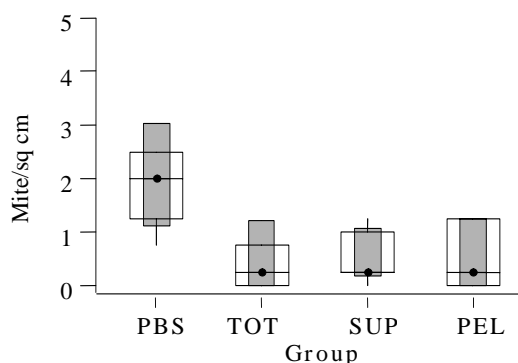


Figure 2. Box-and-whisker plot of mite densities. The solid boxes denote 95% confidential interval (CI) of the median, empty boxes denote internal quarter (IQ) range, the horizontal line with a dot in the middle denotes the median of the total number of mites (all stage of development). Note that unvaccinated control goats (PBS) contained significantly higher number of mites ($P=0.015$) as compared to all vaccinated groups

Measurements of specific antibody by ELISA one week after the third vaccination or at the time of mite challenge revealed that all vaccinated goats had high titre of specific antibodies. The ELISA's OD of serum collected at the end of vaccination schedule increased to over six folds compared to that collected prior to vaccination (Figure 3). Unexpectedly, in the unvaccinated control animals, the titre of antibodies against mites also increased although the increase was much lower. Immunoblot analysis revealed that the banding pattern of these sera was different to that of infested animals (compare lines 1 post vaccination and post challenge, Figure 4). The immunoblot analysis also revealed that vaccination of goats with either mite homogenate, supernatant or pellet elicited antibodies that recognized a large number of mite proteins with molecular weights range from <14.3 to >220 kDa. Sera from naturally infested goats recognised a much smaller number of mite proteins and much less intense

reactions. The most prominent mite proteins recognised by these sera were those with molecular weight above 66 kDa. The sera also recognised more than 10 proteins with molecular weight from 30 to 60 kDa although with less intense reaction. Apparently, no protein with molecular weight less than 30 kDa was recognised by the sera of infested animals.

DISCUSSION

This study shows that vaccination of goats with homogenates prepared from freshly isolated mites conferred protective immunity against mite challenge. The protective immunity was indicated by inhibition of mite population in the vaccinated animals which albeit it was slight but statistically significant. Although the vaccination inhibited significantly the mite population, the severity of lesions, which was used as another indicator of protective immunity, were not affected.

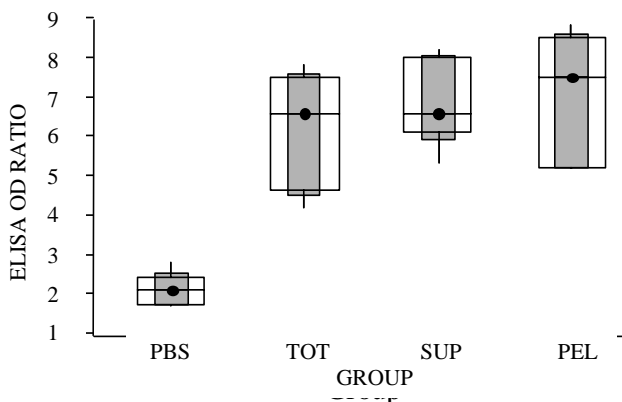


Figure 3. Box-and-whisker plot of ELISA OD RATIO for four groups: PBS, TOT, SUP, and PEL. The solid boxes denote 95% confidential interval (CI) of the median, empty boxes denote internal quarter (IQ) range, the horizontal line with a dot in the middle denotes the median

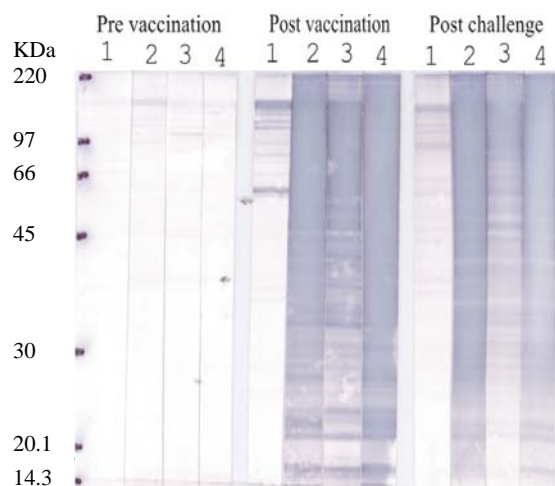


Figure 4. Immunoblot analysis of sera before vaccination, one week after the last (third) vaccination and 8 week after mite challenge. Line 1 represent pooled sera of unvaccinated control (PBS) goats, 2 = Total group (TOT), 3 = Supernatant group (SUP) and 4 = Pellet group (PEL)

This may indicate that mite population is more sensitive as an indicator of protection in the vaccination-challenge experiment than severity of lesions.

In our previous experiments, infestation of goats with approximately 2000 live mites produced scabious lesions that covered almost the entire surface of the infested auricles by 3 weeks, and practically the whole skin by 7 or 8 weeks after infestation (TARIGAN, 2003, 2005; TARIGAN and HUNTLEY, 2005). In the present study, however, infestation of unvaccinated control goats with similar number of mites produced much less severe lesions. In addition, the number of mites per cm² skin surface at the end of experiment were also much lower. The reason for the lower mite population and milder lesions was unknown. The animals were purchased from scabies-free farms and they did not show any signs of mite infestation before the mite challenge. In addition, their sera collected before vaccination did not recognise any scabies protein on ELISA and immunoblot assays. It is, therefore, unlikely that the reasons for the lower mite population and milder lesions in the control goats were specific immunity. The lower sensitivity was also unlikely due to breed differences because the goats used in the present study was the local or Kacang breed, similar to that used in the previous studies.

In our previous studies, vaccination of goats with either soluble- or insoluble-mite-protein fractions failed to confer protective immunity despite the fact that the vaccinations induced strong humoral (IgG) immune responses (TARIGAN, 2005; TARIGAN and HUNTLEY, 2005). The reason for the failure was unknown, it could be associated with inappropriate adjuvant or route of vaccination. Other possibilities include the protective antigens being present in extremely low abundance or

having been degraded or denatured during the extraction or fractionation processes. In the present study, freshly isolated mites were rapidly homogenised and injected into animals in order to prevent protein degradation and denaturation. The fact that vaccination with these fresh mite homogenates conferred protective immunity, albeit it was partial, indicates that the sarcoptic protective antigens are liable to degradation or denaturation.

Although the animals in this study had been vaccinated three times, the vaccinations offered only limited protection. This may indicate that the sarcoptic protective antigens are not only liable to denaturation but also exist in a very low abundance. If this presumption proves to be true, identification and isolation of protective antigens, which constitute the most important steps in the vaccine development, will be very difficult. The conventional approach, in which complex parasite extracts are successively fractionated and the capacity of each fraction to induce protective immunity is determined by vaccination-challenge experiment, seems to be ineffective for *S. scabiei*. The ineffectiveness of this approach seems to be the most probable reason for the failure of identifying the sarcoptic protective antigens in our previous studies (TARIGAN, 2005; TARIGAN and HUNTLEY, 2005). Unlike in *S. scabiei*, the conventional approach has proved to be effective in the isolation of protective antigens from *Boophilus microplus* or *Haemonchus contortus* (KNOX *et al.*, 1999; WILLADSEN *et al.*, 1989). The effectiveness of the approach in those parasites could either be that their protective antigens are more resistant against denaturation, or exist in higher abundance or have higher 'immunoprotectivity'.

Because of the inefficacy of the conventional approach, an alternative approach need to be sought to isolate the sarcoptic protective antigens. The protective antigens would be among the mite proteins that upon attacking by the host immune system resulted in the death or infertility of the mites. These proteins, in addition to having vital function for the mite survival, must be located in such location that could be reached by the effector component of the host immune system. Proteins lining the mite's digestive tract or secreted digestive enzymes may be the best candidates. Targeting only those proteins, rather than the whole mite proteins as in the conventional approach, would be a more feasible approach. Initial studies aim at identifying and characterisation of those proteins are required to facilitate their purification and evaluation of their protective immunogenicity.

To sum up, goats vaccinated with fresh homogenate of *S. scabiei* developed partially protected immunity against mite challenge. The conferred immunity inhibited the mite growth but not the lesion development. The sarcoptic protective antigens are supposed to be liable to degradation and denaturation and exist in very low abundance or low antigenicity. The conventional approach, successively refined fractionation of complex mite extracts and vaccination-challenge experiment, is not appropriate for isolation of the protective antigens.

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