Koch's postulate of *Arcanobacterium pyogenes* and its immunogenicity in local and imported Saanen goats

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Summary

The aim of this survey was to study Koch's postulate of *Arcanobacterium pyogenes* recovered from the necrotic lung of a kid and to compare the immunogenicity of this isolate in local and imported Saanen goats. The disease was successfully reproduced in intrathoracically challenged hamsters which showed lung congestion and liver abscesses, while hamsters that were intraperitoneally challenged showed only the formation of intestinal abscesses. The percentage of histopathologic legions in 12 observed microscopic fields per lung of three groups of hamsters (unchallenged controls, challenged intrathoracically and challenged intraperitoneally) showed a significant increase in lung necrosis of the intrathoracically challenged group, followed by intraperitoneally challenged hamsters, in comparison to unchallenged controls ($p<0.05$). In addition, the frequency of mucus accumulation in alveolar ducts followed the same respective pattern ($p>0.05$), while there was no significant difference in the frequency of neutrophil infiltration ($p>0.05$). The isolate was successfully recovered from the lungs and livers of hamsters challenged by both routes. Saanen does showed significant seroconversion using the indirect haemagglutination (HA) test and slide agglutination test (SAT) and at three weeks following priming and boosting with *A. pyogenes* antigens ($p<0.05$); however, only SAT showed significant seroconversion in local does at three weeks post booster ($p<0.05$). The possible causes and impact of the greater immunogenicity to *A. pyogenes* antigens in Saanen goats compared to local does are discussed.

Keywords

Arcanobacterium pyogenes, Goat, Hamster, Immunogenicity, Koch's postulate, Saanen.

Postulato di Koch sull'*Arcanobacterium pyogenes* e la sua immunogenicità in capre Saanen autoctone e importate

Riassunto

*Lo scopo di questa indagine è stato lo studio dei postulati di Koch sull'*Arcanobacterium pyogenes *evidenziato nella necrosi polmonare di un capretto e la comparazione dell'immunogenicità di questo isolato tra le capre Saanen e quelle locali. La malattia è stata riprodotta con successo nei criceti evidenziando, per via intratoracica, congestione polmonare e ascessi epatici, per via intraperitoneale, solo formazioni di ascessi intestinali. La percentuale di lesioni istopatologiche osservate nei tre gruppi di criceti (animali di controllo non soggetti a inoculazione, animali soggetti a inoculazione intratoracica. animali soggetti a inoculazione intraperitoneale) ha mostrato un aumento significativo della necrosi polmonare e della frequenza di accumulo di muco nei dotti alveolari negli animali con i'inoculazione intratoracica rispetto a quella intraperitoneale e a quelli del gruppo di controllo (p<0,05). Nessuna*

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differenza significativa per la frequenza di infiltrazione dei granulociti neutrofili (p>0,05). L'isolato è stato recuperato con successo dai polmoni e dal fegato dei criceti soggetti a diversa via di inoculazione. Le capre Saanen hanno mostrato significative sieroconversioni con il test di emoagglutinazione indiretta (HA) e il test di agglutinazione su vetrino (SAT), a tre settimane dall'infezione e dal boosting, con l'antigene A. pyogenes *(p<0,05). Solo il test di agglutinazione su vetrino (SAT), a tre settimane post richiamo (p<0,05), ha mostrato una sieroconversione significativa nelle capre locali. Nello studio si discutono le possibili cause e gli effetti della maggiore immunogenicità dell'antigene* A. pyogenes *nella capre Saanen rispetto alla razza locale.*

Parole chiave

Arcanobacterium pyogenes, Capra, Capra Saanen, Criceto, Immunogenicità, Postulato di Koch.

Introduction

The involvement of *Arcanobacterium pyogenes* as an opportunistic pathogen in diseases of goats is well documented in literature (1, 3, 9, 13, 15, 16, 27). In goats, the *A. pyogenes* is one of the most important aetiologies of reproductive tract infection and acts as the aetiologic agent of a secondary infection (8, 13, 16, 18, 27), complicating the incidence of apparent necrotic lesions in the lungs (12, 22, 23), and abscesses on the liver and in the gastro-intestinal tract (2, 23).

Mice were used as a successful model to regenerate the infection by *A. pyogenes* associated with specific pathological lesions (Koch's postulate) (6, 14, 17). To our knowledge, no work has used hamsters in the Koch's postulate studies of *A. pyogenes* and no data exists on the comparison of the immunogenicity of this bacterium in local low productive goats compared to the highly productive Saanen does.

The aim of this work is to study, in hamsters, Koch's postulate of *A. pyogenes* recovered from the necrotic lung of a kid and to compare the immunogenicity of this bacterium in does of local and imported Saanen.

Materials and methods

Isolation and identification of *Arcanobacterium pyogenes*

Sporadic mortalities in kids of Saanen goats, aged 2-4 months, were recorded on different farms located in southern Lebanon, 8-16 km from the border with Israel. The common gross lesions in the dead kids were the presence of necrotic and severely congested lungs, associated with ascites in the abdominal cavity.

A direct smear of the lung on a microscopic slide that was Gram stained, revealed short Gram-positive rods. A pure culture from the lung of one kid appeared on a 5% sheep blood agar that was incubated for 48 h at 37°C.

A pure colony of the culture was subcultured onto another blood agar and incubated similarly. This pure culture had typical colonial and cellular morphology of *Arcanobacterium*. This pure culture was forwarded to the diagnostic laboratory of the American University of Beirut Medical Center (AUBMC), for complete identification using the API system (API Coryne, BioMérieux®, Marcy l'Etoile).

Koch's postulate of *Arcanobacterium pyogenes* in hamsters

The Koch's postulate study of *A. pyogenes* was attempted in hamsters. Hamsters offer a useful animal model to regenerate infection since they are a phylogenetically low classified animals, they are easy to handle and readily available on Lebanese markets.

Briefly, the identified pure isolate of *A. pyogenes* was grown on brain heart infusion (BHI) broth supplemented with 5% newborn calf serum which was incubated at 37°C until the log phase was reached after 3 h. The broth culture was centrifuged at 2 000 × g for 15 min and the pellet was washed twice in saline, resuspended in sterile saline and its density was adjusted using a 'Spectronic 20' spectrophotometer (Bausch and Lomb, New York) to 3% transmittance (T) at 540 nm, corresponding to a bacterial concentration of 1.8×10^7 cfu/ml.

Eight hamsters of the same age were divided into three groups as follows:

- group 1 (two hamsters) was the control with hamsters deprived of challenge
- group 2 (three hamsters), in which each hamster was challenged intrathoracically between the last two ribs with 0.1 ml of the 3% T-adjusted density of *A. pyogenes*
- group 3 (three hamsters), in which each hamster was challenged intraperitoneally with the same dose as that used for the hamsters of group 2.

All hamsters were sacrificed under CO_2 gas at three days post challenge as described above. Gross lesions in the visceral organs were recorded and cultures on blood agar for the liver and lungs of each hamster were performed. Lung specimens were kept in 10% formalin before despatch to the pathology department of the AUBMC for sectioning and preparation of hematoxylin and eosin (H&E) stained tissues (21). Microscopic lesions were recorded and quantitated in 12 fields/lung/hamster.

Acquired humoral response to *Arcanobacterium pyogenes*

Preparation of *Arcanobacterium pyogenes*/chicken red blood cell emulsion

The preparation of the aqueous-antigens phase of *A. pyogenes* to be incorporated in an oil emulsion was performed according to Barbour *et al.* (5). Briefly, the *A. pyogenes* culture was washed in sterile saline and finally reconstituted to 1.5% transmittance (T) at 540 nm. The inactivation of the viable cells was performed using 0.66% formalin and confirmed by the absence of growth on blood agar. Tween 80 was added to the inactivated aqueous phase at a ratio of 1:1 000 (v/v); 25% washed chicken red blood cells (c-RBC) were inactivated from any contamination using the above-mentioned procedure. Confirmation of sterility was performed, followed by addition of Tween 80 at the above ratio. The inactivated *A. pyogenes* and the prepared c-RBC were mixed at 1:1 (v/v). The sterile oil phase (4.4 ml Arlacel C + 100 ml mineral oil) was prepared and mixed with the aqueous phase of *A. pyogenes*/c-RBC antigens, forming a stable emulsion.

Sensitisation of does with *Arcanobacterium pyogenes* chicken red blood cell emulsion

The sensitisation of does (local and Saanen breeds) using the *A. pyogenes*/c-RBC emulsion was performed as described below.

A total of 12 non-pregnant does were included in the design (six local and six Saanen goats). The local and the Saanen groups were each divided into two subgroups (three does/subgroup), resulting in four subgroups namely:

- local controls (deprived of emulsion administration)
- local sensitised (emulsion administered)
- Saanen controls (deprived of emulsion administration)
- Saanen-sensitised (administered the emulsion).

Each doe in the two sensitised groups received the first 4 ml of the emulsion (priming) subcutaneously in the neck, while the second 4 ml of the emulsion was administered as a booster 4 weeks later, via the same route.

Plasma samples were collected from the jugular vein of all does at priming, boosting and 3 weeks following the booster. The plasma was kept at –20°C until further analysis.

Quantitation of the humoral response

The quantitation of the humoral response in the 12 does, specific to *A. pyogenes*, in the presence of numerous competitive antigens of the c-RBC, was performed using two procedures, as follows:

- indirect haemagglutination (HA) titration against human-RBC (h-RBC) sensitised with soluble antigens of *A. pyogenes*
- slide agglutination test (SAT) titration against cell-free particulates of *A. pyogenes*.

Indirect haemagglutination titration

The indirect HA titration was performed as described by Shirai *et al.* (26). Briefly, the h-RBC were washed and adjusted to 20% suspension in phosphate buffered saline (PBS). Glutaraldehyde was added at 0.2%. A pellet of *A. pyogenes* was suspended to 20% (w/v) in

PBS and an equal volume of ethyl ether was added to it. Dialysed antigens of *A. pyogenes* were placed with glutaraldehyde-treated h-RBC and the suspension was adjusted to 0.5%. Each plasma sample was subjected to serial dilution (with a titration dilution factor of 0.5) in PBS and each dilution was placed in a microtitre plate, in 50 µl volume, with 50 µl volume of the *A. pyogenes* sensitised h-RBC for a period of 18 h. The maximum dilution of the plasma sample that results in complete agglutination was recorded as the titre for that sample.

Slide agglutination titration

The serially diluted plasma in PBS was each placed in a 50 µl volume on a microscopic slide with 50 µl of 3% adjusted transmittance of cell-free particulates of *A. pyogenes* (l = 540 nm).

The maximum dilution of the plasma sample that resulted in apparent particulate agglutination of the *A. pyogenes* cells was considered the SAT titre.

Statistics

The percentages of specific lesions in each visceral organ were compared statistically between the three hamster groups included in the Koch's postulate study using Chi-square ($p = 0.05$).

The Chi square was also used to compare the percent recovery of *A. pyogenes* from each visceral organ (liver and lung) of each of the three groups of hamsters included in the study.

A similar statistical procedure was used to compare the percent frequency of presence of a specific microscopic observation of the H&E stained tissue ($p = 0.05$) in the three groups of hamsters.

Analysis of variance (ANOVA) was used to compare the mean indirect HA titres and the mean SAT titres specific to *A. pyogenes* at the three times (priming, booster and 3 weeks post booster). The means were compared to evaluate the significance of seroconversion in each breed alone, and to evaluate the immunogenicity of *A. pyogenes* between the two breeds at different times following priming and boosting.

Results and discussion

The data relative to the Koch's postulate study of *A. pyogenes* recovered from a goat kid are presented in Figs 1, 2 and 3. The two different challenge routes administered to the hamsters resulted in different frequencies of gross lesions in the lungs, liver and intestines (Fig. 1). More specifically, the *A. pyogenes* isolate was able to reproduce gross lesions only in intrathoracically challenged hamsters, with 3/3 and 1/3 of the animals showing lung congestion ($p<0.05$) and liver abscesses ($p>0.05$), compared to a total absence of such lesions in the controls. However, the reproduction of the gross lesions in intraperitoneally challenged hamsters was confined to the formation of intestinal abscesses in 3/3 of the animals compared to its absence in controls ($p<0.05$). The difference in frequencies of organs revealing specific gross lesions, in relation to challenge route, is linked to the difference in the structure and function of the lymphatic system in the thoracic and

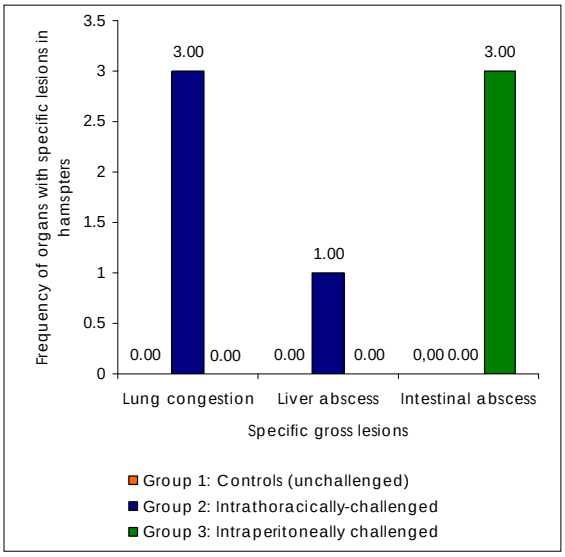


Figure 1
Frequency in three respective hamster organs showing specific gross lesions following an intrathoracic or intraperitoneal challenge with *Arcanobacterium pyogenes*
The frequency of lung congestion was significantly highest in group 2 (intrathoraciacally challenged) ($p<0.05$), while the highest percentage of intestinal abscesses was observed in intraperitoneally challenged hamsters ($p<0.05$)
Controls had no lesions

peritoneal cavities of the body (10, 11). Previous studies have documented differences in the frequency of gross lesions and reproduction of the disease based on the nature of the challenge route (10, 11), affected by immune reactions that differ at different parts of the lymphoid system.

The frequency of recovery of *A. pyogenes* from the lungs and liver of two groups of hamsters, challenged intrathoracically and intraperitoneally, is presented in Figure 2. There was a similar frequency of recovery of *A. pyogenes* in the lungs of the two groups (1/3), with a higher recovery frequency in the livers of intrathoracically challenged hamsters (3/3) compared to the recovery from the livers of intraperitoneally challenged animals (2/3) (p>0.05). None of the livers or lungs of the controls revealed the presence of *A. pyogenes*. The higher frequency of recovery of this organism in both groups, from the livers compared to the lungs, could be due to a higher liver tropism of this bacterium compared to its tropism to the lungs of hamsters. Previous studies on *A. pyogenes* have shown a higher recovery frequency of this organism from liver compared to peritoneal fluid and blood (14). Indeed, the receptors in the hepatocytes of the livers to *A. pyogenes* are more abundant than those present in the lung tissue (7, 14, 24).

The percentage of 12 observed microscopic fields per lung, showing a specific pathological change in the three groups of hamsters subjected to Koch's postulate, is presented in Figure 3. There was a significant increase in the percent lung necrosis of intrathoracically challenged hamsters (69.4%), followed by those challenged intraperitoneally (44.4%) in comparison to the unchallenged controls (20.8%) (p>0.05). In addition, the frequency of mucus accumulation in the alveolar ducts present in the 12 observed microscopic fields was the highest in intrathoracically challenged hamsters, equivalent to 36.1%, compared to 30.5% in the intraperitoneally challenged hamsters and 4.2% in controls (p>0.05).

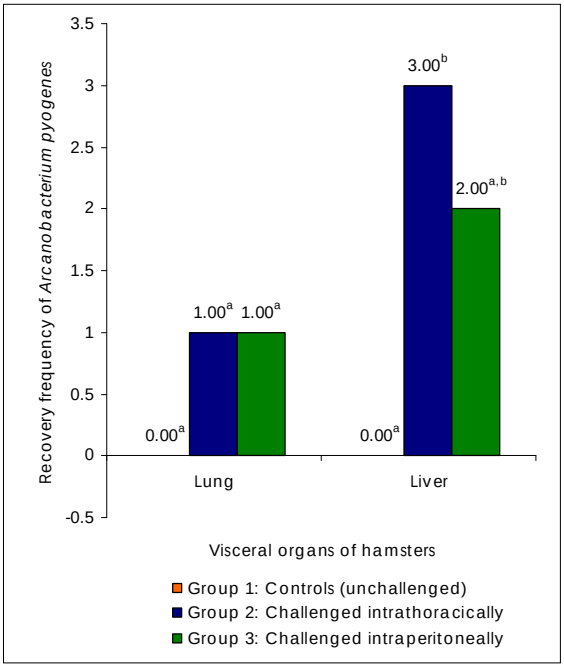


Figure 2
Frequency of recovery of *Arcanobacterium pyogenes* from the lungs and livers of three hamsters challenged intrathoracically or intraperitoneally
The superscripts reveal differences in the recovery percentage of *A. pyogenes* from the respective organs (p<0.05)

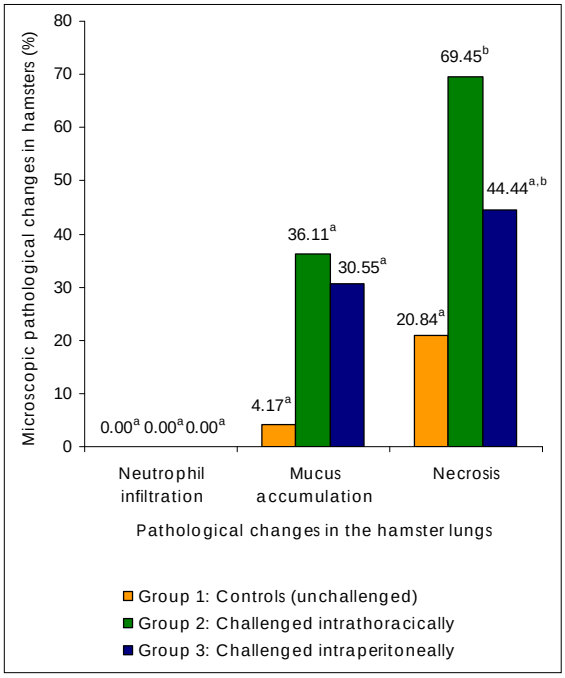


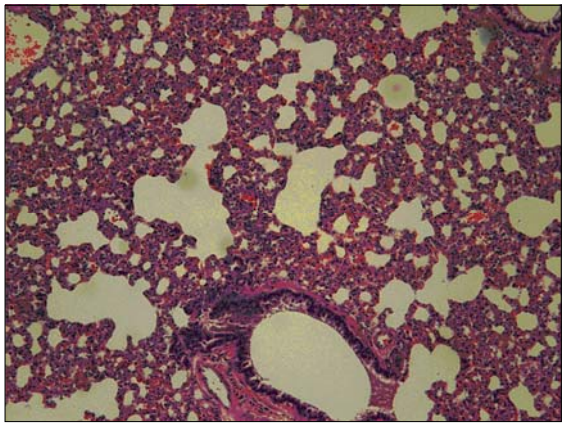
Figure 3
Percentage of microscopic fields showing specific pathological changes in the lungs of the three groups of hamsters
The superscripts reveal differences in the frequency of occurrence of a specific microscopic pathological lesion

There was an absence of neutrophil infiltration in the lungs of hamsters of the three groups (p>0.05) indicating early stages of tissue necrosis. The intrathoracic route of challenge appears to have the greatest capacity to induce microscopic necrosis in the parenchyma and mucus cells of the lungs, associated with mucus accumulation in the air passages (Fig. 3) and in the higher success of recovery of *A. pyogenes* from the liver (Fig. 2) and in the induction of the highest frequency of gross lung congestion (Fig. 1). Representative images of the three microscopic lesions are provided in Figure 4. The data from Figures 1, 2 and 3 will possibly encourage the use of the intrathoracic route of challenge in hamsters for future investigations on the comparison of pathogenesis of different strains of *A. pyogenes* recovered from goats, as it proved its capacity to reproduce the disease using Koch's postulate.

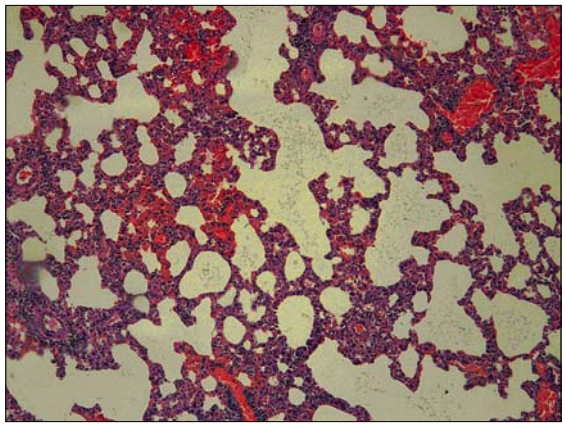
The data related to the quantitative comparison of immunogenicity of *A. pyogenes* in local versus imported Saanen does are presented in Figures 5 and 6.

The differential mean titre is the mean titre of vaccinated hamsters minus the mean titre of controls at a given time to *A. pyogenes*-sensitised h-RBC (*A. pyogenes*-h RBC) in local versus Saanen does as shown in Figure 5. The immune system of the local does failed to respond to the injected killed antigens of *A. pyogenes* at 4 weeks post priming, and even at 3 weeks post boosting with the same antigens. However, the Saanen goats had a mean differential HA titre to *A. pyogenes* carried on the surface of h-RBC at priming which was equivalent to 1.3, increasing to 10.7 at 4 weeks post priming and to a higher seroconverted mean titre of 21.3 at 3 weeks post boosting (p<0.05). This superiority in acquired immunity to *A. pyogenes* by the Saanen compared to local does (p<0.05) is most likely due to presence of *A. pyogenes* clones of B-cells and T_H cells that have respective receptors of IgM/IgD and T-cell receptors (24, 25) specific to antigens of *A. pyogenes*. The presence of such receptors would have helped in the recognition of the bacterium and in acquiring a immune response. The absence of

A) Absence of neutrophil infiltration in the lungs of hamsters



B) Mucous accumulation in the bronchial tree of the hamster's lungs



C) Necrosis in the lung of the hamster

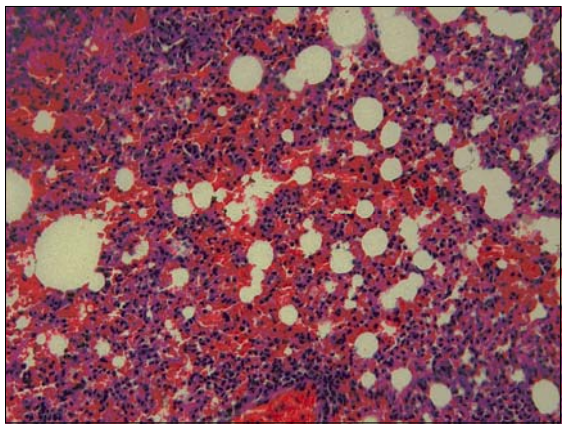


Figure 4
Microscopic lesions in the lungs of hamsters challenged with *Arcanobacterium pyogenes*

an immune response in the local does to such a bacterium is most likely due to absence of specific clones (6, 20), or due to their lower immunogenicity compared to that of the Saanen breed which are renowned for their high higher immunogenicity (19).

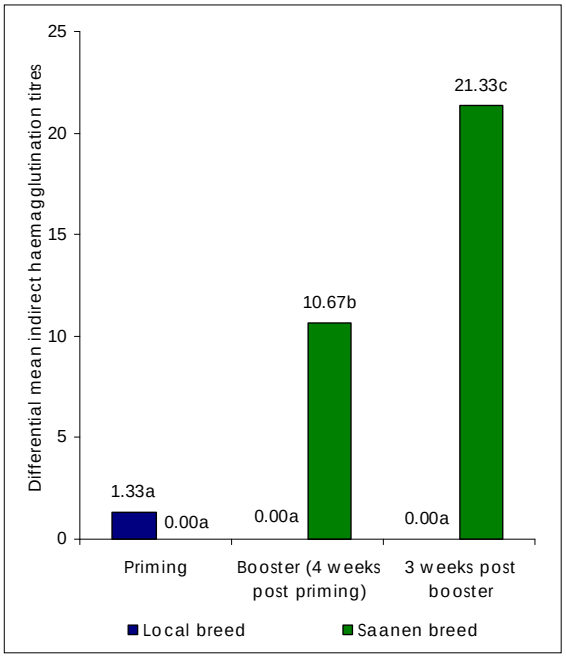


Figure 5
Comparison of differential mean indirect haemagglutination titre to *Arcanobacterium pyogenes*-sensitised human red blood cells in local and Saanen does
The differential mean titre is the mean titre of vaccinated hamsters minus the mean titre of controls at a given time
The superscripts reveal significant differences in differential mean indirect haemagglutination titres (p<0.05)

This elevated pattern of seroconversion in Saanen goats to soluble antigens of *A. pyogenes* presented on h-RBC was confirmed by another quantitative test, using titration of plasma samples and the reaction of each dilution using the SAT (Fig. 6). This quantitative test measures the titre, i.e. the maximum dilution of the plasma sample that remains with adequate antibodies to specific erythrocyte sensitising substance (ESS) of *A. pyogenes*, to cause agglutination (4). The pattern of superior seroconversion to *A. pyogenes* in Saanen goats compared to local does was deduced from the SAT data of Figure 6, showing a significant higher mean differential SAT titre in Saanen compared to local does at 3 weeks post booster (p<0.05).

This similarity in the seroconversion patterns deduced from the data of mean differential indirect HA titre to soluble *A. pyogenes* presented on h-RBC and from the SAT is most likely due to correlation of the two methods.

The analysis of regression data by both methods revealed an equation of y = 1.6x–17.5 (y = mean titre quantitated by SAT, and x = mean titre quantitated by indirect HA), with R^2 = 0.665. The y and x were highly correlated at p<0.05.

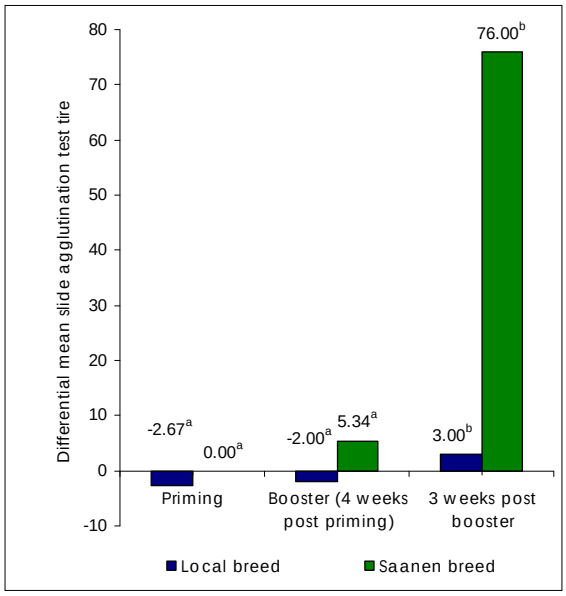


Figure 6
Comparison of differential mean slide agglutination test titre to cell-free particulates of *Arcanobacterium pyogenes* in local and Saanen does
The differential mean titre is the mean titre of vaccinated hamsters minus the mean titre of controls at a given time
The superscripts reveal significant differences in differential mean slide agglutination titres (p<0.05)

Conclusions

Using Koch's postulate to recover *A. pyogenes* from a kid in hamsters is more successful using intrathoracic challenge in comparison to the intraperitoneal route, and the significant correlation between the two quantitative tests used in this study helped to reveal the superiority of seroconversion s *A. pyogenes* in Saanen goats in comparison to local does.

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