Acta vet. scand. 1989, *30*, 275–283.

Corynebacterium Pseudotuberculosis Infection in Goats VIII.

The Effect of Vaccination against Experimental Infection

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Holstad G., J. Teige jr. and H. J. Larsen: Corynebacterium pseudotuberculosis infection in goats VIII. The effect of vaccination against experimental infection. Acta vet. scand. 1989, 30, 275–283. – The effect of an inactivated vaccine against *C. pseudotuberculosis* infection was tested on castrated male kids from a herd free from caseous lymphadenitis. The animals were divided into 3 groups with 8 animals in each. Group 1 was immunized with crude filtrated *C. pseudotuberculosis* toxoid and whole killed organisms, while Group 2 in addition was given levamisole. The kids were vaccinated twice at an interval of 4 weeks. Group 3 consisted of unvaccinated animals. All groups were challenged subcutaneously with live bacteria 4 weeks after the last vaccination.

Unvaccinated animals showed the most severe course of illness after challenge.

Development of abscesses in the regional lymph nodes (*lnn. subiliaci*) was significantly more common in unvaccinated than in vaccinated kids at necropsy 2 months after challenge. There was, however, no such difference between the vaccinated groups, and there was no difference between any of the groups as regards abscess formation at the inoculation site.

In each of the 2 vaccinated groups, there was a titre rise following vaccination in the hemolysis inhibition test, whereas no such rise was seen in the bacterial agglutination test. The titre values in both tests increased significantly after challenge in all the groups, the increase being most rapid in the vaccinated animals.

The present investigation indicates that development of caseous lesions in lymph nodes in goats, following subcutaneous inoculation with *C. pseudotuberculosis,* can be reduced by an inactivated vaccine containing whole organisms and crude toxin.

whole killed bacteria; crude filtrated toxoid; levamisole; bacterial agglutination test; hemolysis inhibition test; challenge; abscesses.

Introduction

Caseous lymphadenitis, caused by *Corynebacterium pseudotuberculosis,* is a chronic disease in sheep and goats. Abscess formation usually occurs in lymph nodes, the thick wall of the lesions rendering the abscesses refractory to antibiotic treatment.

Caseous lymphadenitis is a worldwide problem, and control of the disease has been a major concern to the sheep and goat industry. The disease is difficult to eradicate once it has become endemic. Due to difficulties in controlling the disease, many studies on the mechanisms of protective immunity have been carried out with the ultimate aim of developing effective vaccines (*Jolly* 1965, *Cameron & Minnaar* 1969, *Cameron et al.* 1969, *Cameron & Engelbrecht* 1971, *Cameron & Purdom* 1971, *Cameron et al.* 1972, *Irwin & Knight* 1975, *Zaki* 1976, *Cameron*

1977, *Nairn et al.* 1977, *Tashjian & Campbell* 1983, *Batey* 1986a). The immunity appears to be complex, and it has been attributed to both cell-mediated and humoral mechanisms (*Batey* 1986b). There are apparently two factors, cell wall lipid (*Carne et al.* 1956) and exotoxin (*Carne* 1940), that play essential roles in the development of lesions. The lipid allows the microorganism to resist digestion by cellular enzymes and to persist as a facultative intracellular parasite even in activated macrophages (*Hard* 1972). It has been suggested that the cell wall lipid is the factor involved in the formation of abscesses, while the exotoxin is responsible for spread of the organisms (*Zaki* 1976).

Several vaccination trials against *C. pseudotuberculosis* infection have been carried out in sheep and goats. *Cameron* (1972) found that inactivated vaccines did not prevent development of abscesses following i.v. inoculation of the organism, and suggested the use of a live vaccine against caseous lymphadenitis. However, inactivated vaccines have been shown to have some protective effect against subcutaneous or intradermal infection both in sheep and goats (*Nairn et al.* 1977, *Burrell* 1978, *Nairn* 1981, *Anderson & Nairn* 1984, *Brown et al.* 1986, *LeaMaster et al.* 1987). Protective effect of antitoxins has been reported (*Nairn et al.* 1977, *Burrell* 1978, *Brown et al.* 1986), though other antigens may also be involved (*Burrell* 1983). *Cameron & Purdom* (1977) considered that protection against *C. pseudotuberculosis* infection in mice could depend upon a complex configuration of antigens which might include the toxin. *Brogden et al.* (1984) demonstrated some protection following i.v. inoculation of the organism in lambs which had been vaccinated with whole cells or cell walls.

The purpose of the present investigation was to evaluate the efficacy of an inactivated vaccine against experimental *C. pseudotuberculosis* infection in goats. The vaccine contained whole killed bacteria and crude filtrated toxoid. In addition, a study was made on the possible role of levamisole in enhancing the efficacy of the vaccine.

Material and methods

Animals

The investigation included 24 castrated male kids which were 1 month old when the experiment started. The kids came from a herd in which clinical and serological examinations of animals had indicated the absence of caseous lymphadenitis.

The kids were placed in thoroughly cleaned and disinfected pens. They were fed an acidified milk substitute, hay, and pelleted calf concentrates, as well as supplementary minerals and water.

Vaccine

The vaccine was prepared from a strain (NVH 2586*) of *C. pseudotuberculosis* isolated from goat. The strain had been shown to cause lesions following subcutaneous inoculation in goats (*Holstad & Teige* 1988a).

Whole killed organisms (Part A). Cultivation was carried out on brain heart infusion broth (Difco) (250 ml) on a shaker for 2 days at 37°C. The bacteria were washed 3 times in phosphate buffered saline (PBS) before addition of formalin to a final concentration of 0.5 %, and then stored 5 days at 4°C. Inactivation of the cells was checked by culture on blood agar. The killed bacteria were freeze-dried, stored at room temperature and then mixed with an adjuvant described by *Fodstad* (1980). Freeze-dried bac-

* Culture collection at the Department of Microbiology and Immunology, Norwegian College of Veterinary Medicine, Oslo.

teria were suspended in equal parts of olive oil and liquid paraffin to give a concentration of 4 mg freeze-dried material per ml. Pumice powder was added to the suspension to give a concentration of 6.6 mg per ml. The adjuvant referred to was chosen because it was found to give a more homogenous suspension of bacteria than Freund's incomplete adjuvant.

Crude filtrated toxoid (Part B). Cultivation was carried out on brain heart infusion broth (2 litres) for 7 days at 30°C. The culture was centrifuged and the supernatant filtered through a millipore filter (0.22 µm). The toxin titre was 5,4 ($\log_{10}$ reciprocal value) using the method described by *Holstad* (1986). The toxin was inactivated by adding formalin to a final concentration of 0,5 % and storing the filtrate for 3 months at room temperature. The crude toxoid was then stored at 4°C before use. Crude filtrated toxoid was then suspended in equal parts of Freund's incomplete adjuvant.

Vaccination

The animals were divided into 3 groups with 8 kids in each. Kids in Groups 1 and 2 were vaccinated with 1 ml of Part A and 2 ml of Part B. All the immunized animals were injected twice, at an interval of 4 weeks. The vaccine was applied subcutaneously behind the shoulder on both sides. Each kid in Group 2 received, in addition, about 2 mg/kg levamisole in tablet form every other day from the day of vaccination until 1 week before challenge. Kids in Group 3 were used as controls, no treatment being given.

Challenge

Before challenge the animals were moved to 2 thoroughly cleaned and disinfected pens. Four kids from each group were placed together in each pen. Strain 2586 was used for challenge, and the bacterial suspension was prepared as described by *Holstad & Teige* (1988b). Each animal was given 1 ml of this bacterial suspension 4 weeks after the last immunization. The suspension was applied subcutaneously 1 cm below the right *lnn. subiliaci* as described by *Holstad & Teige* (1988a).

Serological examinations

Blood samples for serological examinations were collected from all the kids on the first vaccination and 1, 3, 4, 6, 7, 8, 9, 10, 11, 13, 15, and 16 weeks later. Sera were prepared according to standard procedures and stored at –20°C. All the sera were examined for antibodies to *C. pseudotuberculosis* using the bacterial agglutination test (BAT) and the hemolysis inhibition test (HIT) (*Holstad* 1986). Titre values were expressed as $\log_{10}$ reciprocal value of the highest positive serum dilution in the tests. A positive titre (T) was stipulated as $T \geq 2.1$ in BAT and as $T \geq 0.6$ in HIT (*Holstad* 1986).

Clinical examinations

The animals were examined weekly following the vaccinations.

The temperature of each animal was measured daily the first 6 days after challenge. In addition, the animals were inspected and palpated for lesions daily the first 3 days after challenge.

Post mortem examinations

Two months after challenge the animals were killed, weighed and a necropsy performed.

Sections for histological examination were collected from the *lnn. subiliaci* regional to the inoculation site. Some sections of the opposite *lnn. subiliaci* were also collected in order to obtain a basis for lymph node evaluation. All the sections were stained with hematoxylin and eosin.

Samples for bacteriological examinations were taken from abscesses. Cultivation was carried out on blood agar plates, the plates being incubated for 48 h at 37°C in 10 % CO_2 atmosphere. Bacterial colonies suspected to be *C. pseudotuberculosis* were identified according to *Buchanan & Gibbons* (1974).

Statistical analysis

Statistical calculations were carried out using Wilcoxon Signed Ranks Test and Yates' correction for continuity. $p \leq 0.05$ was taken to indicate statistical significance.

Results

Serological examinations

Fig. 1 shows the sequential titre values in BAT and HIT.

BAT-titres showed a similar course in the 2 vaccinated groups during the investigation period. No increase in titre was detected following immunization. However, during the week after challenge, BAT-titres increased significantly, all the animals being positive in the test. In unvaccinated animals, a moderate rise in the BAT-titre was observed the first week after challenge, while during the second week, the titres increased to significantly higher values than in the vaccinated groups. In all the groups, the BAT-titres peaked during the first 1–2 weeks following challenge, decreasing then to significantly lower values during the following weeks.

The course and level of HIT-titre was similar in the 2 vaccinated groups during the investigation, the level being slightly lower in Group 2. A slight increase in titre was demonstrated following the second immunization, 6 animals in Group 1, and 3 animals in Group 2 being seropositive. The titres increased significantly during the first week following challenge but did not peak until several weeks later in both the vaccinated groups. In the unvaccinated group, the HIT-titre increased during the period 2–5 weeks after challenge. The values were, however, significantly lower than in the vaccinated groups. All the unvaccinated animals were seropositive first 8 weeks after challenge.

Clinical examinations

Small swellings (1–2 cm) were seen at the vaccination sites a few days following immunization. Fistulous formation in the swellings was not recorded.

Average temperature on the day after challenge was 40.8, 40.9 and 41.0°C for animals in Groups 1, 2, and 3, respectively. The temperature decreased to normal values 4 days after challenge in the vaccinated animals. In unvaccinated animals, however, the temperature decreased more slowly.

Within a day after challenge, each kid developed swellings at the inoculation site and in the regional lymph node. Pus formation appeared at the inoculation site 2 days after challenge in most of the animals. The kids showed signs of discomfort on palpation of the lesions. In 4 of the control animals, oedema appeared over the abdomen on days 2 and 3 after challenge, while no such reaction was observed in vaccinated animals.

Postmortem examinations

At sacrifice, the kids' body weight varied between 18 and 27 kg, the mean weights being 22.7, 22.6 and 23.3 kg for animals in Groups 1, 2 and 3, respectively.

Abscess formation demonstrated by necropsy of animals in the different groups are presented in Table 1. Abscess size at the inoculation site was largest among the kids in the control group. There was otherwise no significant difference between the three groups of animals concerning the number of abscesses found in this location. Abscesses at the

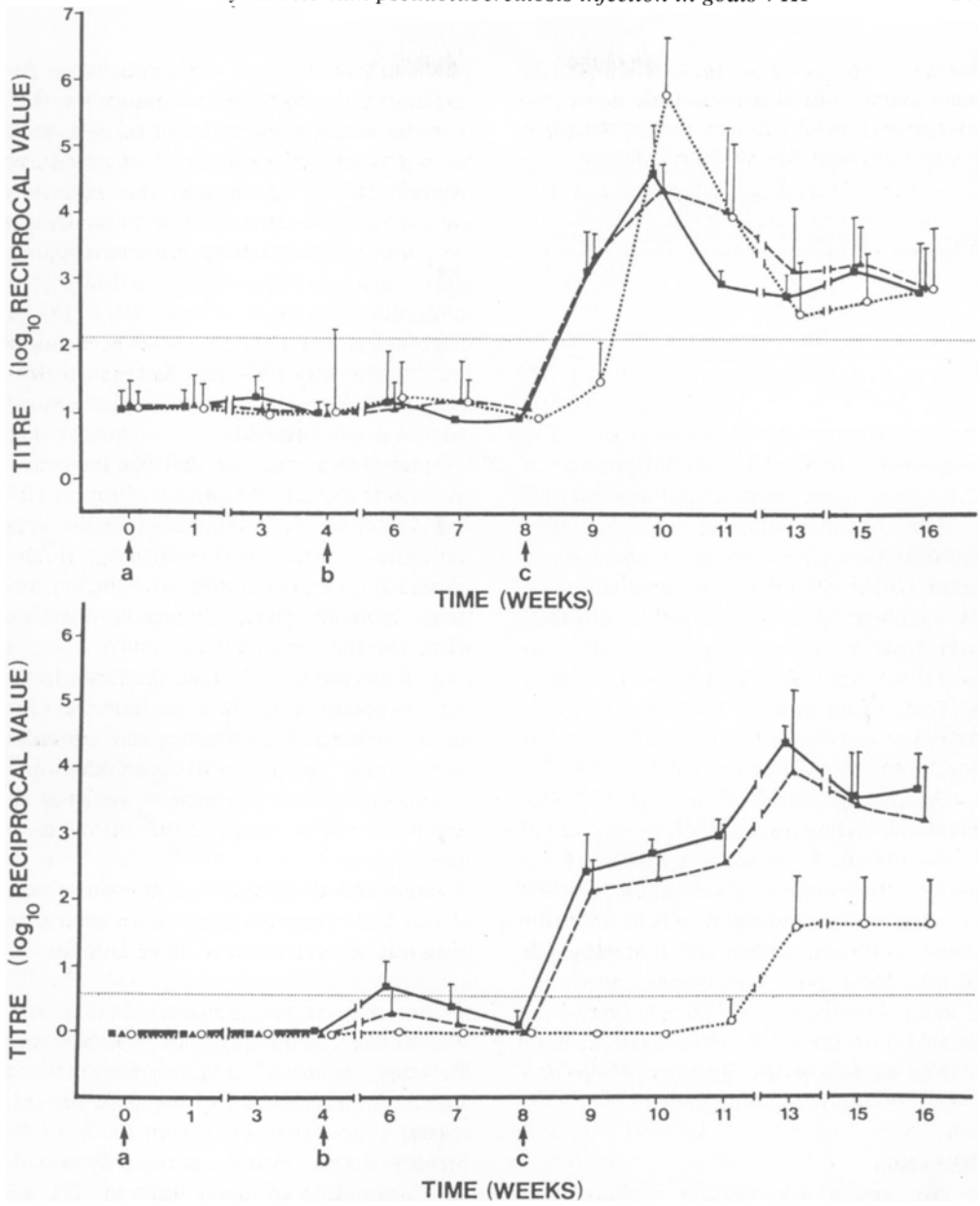

Figure 1. Titres (means and standard deviations) for antibodies against C. pseudotuberculosis in the bacterial agglutination test (top) and the hemolysis inhibition test (bottom). Horizontal dotted lines indicate the limit between positive and negative titres.

Group 1 (vaccinated with whole killed organisms and crude filtrated toxoid)

Group 2 (vaccinated with whole killed organisms and crude filtrated toxoid and given levamisole)

Group 3 (no vaccine and no levamisole)

a) Primary immunization, b) Secondary immunization. c) Challenge.

Table 1. Presence of abscesses at postmortem examination 2 months after challenge in vaccinated (Groups 1 and 2) and unvaccinated (Group 3) animals. Each group includes 8 animals.

Group	Number of animals with abscesses at		
	inoculation site	lnn. subiliaci*)	other sites
1	4	0	0
2	5	2	1
3	6	8	2

*) Regional lymph node.

inoculation site were found in 5 out of 9 vaccinated animals that were all positive in HIT following vaccination, and in 4 out of 7 vaccinated animals negative in this test after immunization. Development of abscesses in lymph nodes regional to the inoculation site was significantly more common in unvaccinated than in vaccinated animals, while no such difference could be observed between the 2 vaccinated groups. The regional lymph nodes showed, however, a marked swollen appearance in most of the animals. The histological examination of these lymph nodes revealed a picture as described by *Holstad & Teige* (1988a). A broad cortex with an increase in the number of secondary follicles, a moderate proliferation of cells in the paracortex area, and plasma cell infiltration in the medullary cords, were demonstrated.

C. pseudotuberculosis in pure culture was isolated from the 18 abscesses examined, 15 of these were from the inoculation sites and 3 from the regional lymph nodes.

Discussion

In the present investigation, unvaccinated animals showed a more severe course of illness after challenge than the vaccinated ones. Abscesses in the lymph nodes regional to the inoculation site were significantly more common in unvaccinated than in vaccinated kids 2 months after challenge.

Whole cells and toxoid were included in the vaccine in the light of the possibility that both the exotoxin and cell wall antigens may be important for development of immunity (*Burrell* 1983). Levamisole, a commonly used anthelminticum, has been shown to have immunopotentiating properties, apparently due to stimulation of cell-mediated immunity (*Renoux & Renoux* 1972, 1973), macrophage function (*Hoebeke & Franchi* 1973, *Lima et al.* 1974) and non-specific immunity (*Fisher et al.* 1974). Investigations carried out in mice have also revealed that levamisole-treatment can enhance the resistance to *C. pseudotuberculosis* infection (*Irwin & Knight* 1975). However, in the present study, there was no difference in the immunity achieved following infection between animals given levamisole together with vaccine and animals given vaccine only. *Cameron* (1977) found that levamisole had no potentiating effect on immunity induced in sheep by an inactivated *C. pseudotuberculosis* vaccine, and concluded that levamisole was of no practical value as an immunostimulant against the infection in normal sheep.

A response following vaccination was observed in HIT. Animals given levamisole gave a slightly weaker response in HIT following vaccination and challenge, though the difference was too small to conclude that levamisole suppress the production of antitoxin. However, depressed immunoglobulin levels after levamisole treatment has been demonstrated in mice (*Irwin & Knight* 1975). In the present study, several vaccinated animals had measurable antitoxin titres in HIT before challenge exposure. Antibodies to the exotoxin produced after vaccination may have limited the local spread and general dissemination of *C. pseudotuberculosis* in the initial bacterial multiplication period following infection.

In BAT, no response could be detected after vaccination. *Burrell* (1983) reports that the important cellular antigen cannot be assayed by the agglutination test even though high protection can be achieved by immunization with cells alone. The present study showed, however, that vaccination had some effect on the titres in BAT, antibodies being detected 1 week earlier in vaccinated than in unvaccinated animals after challenge. The BAT-titre in the unvaccinated group increased to about the same value as described by *Holstad & Teige* (1988a) 2 weeks after infection. These results indicate that a significantly higher BAT-titre can be detected 2-3 weeks following infection than later in the course of the disease.

The inflammatory reaction at the inoculation site during the first days following infection was most prominent in unvaccinated kids. Presence of antitoxin probably had a protective effect against the local inflammation arising following inoculation. However, the presence of such antibodies had no influence on abscess formation at the inoculation site. Other investigations have shown that inactivated vaccines cannot prevent formation of suppurative lesions at the site of challenge (*Nairn et al.* 1977, *Anderson & Nairn* 1984, *LeaMaster et al.* 1987). A more restrictive bacterial proliferation at the inoculation site may have contributed to formation of smaller abscesses in the vaccinated than in the unvaccinated animals in the present study. Inflammation enhances the multiplication of *C. pseudotuberculosis* (*Batey* 1986a), and antitoxins may thus have contributed to the formation of smaller abscesses at the inoculation sites in the vaccinated animals by reducing the inflammatory activity of the exotoxin.

The present investigation indicates that development of caseous lesions in lymph nodes following subcutaneous inoculation with *C. pseudotuberculosis* can be reduced by an inactivated vaccine containing whole organisms and crude toxin. Trials should be carried out to detect the efficacy of the vaccine used in the present study against natural *C. pseudotuberculosis* infection in goats.

Acknowledgement

The investigation was supported by grants from the Agricultural Research Council of Norway.

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Sammendrag

Corynebacterium pseudotuberculosis infeksjon hos geit VIII. Effekten av vaksinasjon mot eksperimentell infeksjon.

Effekten av en inaktivert vaksine mot eksperimentell infeksjon med *C. pseudotuberculosis* ble undersøkt hos kastrerte bukkekje fra en besetning fri for kaseøs lymfadenitt. Dyrene ble delt i 3 grupper med 8 kje i hver. Gruppe 1 ble immunisert med filtrert urenset toksoid av *C. pseudotuberculosis* og hele drepte bakterier, mens gruppe 2 fikk levamisol i tillegg til dette. Dyrene ble vaksinert 2 ganger med 4 ukers mellomrom. Gruppe 3 bestod av uvaksinerte dyr. Alle 3 grupper ble belastet subcutant med levende bakterier 4 uker etter siste vaksinasjon.

Uvaksinerte dyr fikk de mest alvorlige sykdomsforløpene etter belastning. Abscesser i de regionale lymfeknutene (*lnn. subiliaci*) til inokuleringsstedet ble påvist hos signifikant flere uvaksinerte enn vaksinerte kje ved obduksjon foretatt 2 måneder etter vaksinering. Det var imidlertid ingen forskjell mellom de vaksinerte gruppene i forekomst av dyr med slike abscesser. Abscessdannelse på inokuleringsstedet forekom like vanlig hos dyr i alle grupper.

Etter vaksinering ble det påvist økning i titer ved antihemolysintest i de vaksinerte gruppene. Det ble imidlertid ikke påvist økning i titer ved bakterieagglutinasjonstest etter vaksinering. I alle gruppene økte titerverdiene ved begge testene signifikant etter belastning, raskest hos de vaksinerte.

Foreliggende undersøkelse tyder på at en vaksine som inneholder hele drepte bakterier og urenset toksoid, kan redusere dannelsen av abscesser i lymfeknuter etter subcutan inokulering av *C. pseudotuberculosis* hos geit.

(Received June 12, 1988; accepted October 17, 1988).

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