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From the Department of Veterinary Virology and Immunology and the Department of Pathology, Royal Veterinary and Agricultural University, Copenhagen, Denmark.

BOVINE MASTITIS INDUCED BY A COMMON INTESTINAL CHLAMYDIA PSITTACI STRAIN

A PATHOGENETIC AND SEROLOGICAL INVESTIGATION*

By

L. Rønsholt and *A. Basse*

RØNSHOLT, L. and A. BASSE: *Bovine mastitis induced by a common intestinal Chlamydia psittaci strain. A pathogenetic and serological investigation.* Acta vet. scand. 1981, 22, 9—22. — Cattle are frequently infected with a persisting intestinal Chlamydia psittaci strain, which through the manure deposit will contaminate their surroundings. The potential of such a strain (ROS) for inducing mastitis was demonstrated in 7 cows, inoculated in 1 udder-quarter through the teat canal, and the pathogenetic events provoked were compared with those of more virulent and infrequently isolated strains, viz. the EAE and the SBE strains. The chlamydial agent caused during a 2—3 weeks period a local, self-limiting exudative mastitis with a fibrinous secretion, leading to a state of reduced milk production. Increasing titres of complement fixing and agglutinating antibodies were demonstrated in whey and serum. The results from the 2 serological methods did not substitute each other entirely, so that both tests had to be employed in order to demonstrate the presence of the agent in all cases. Demonstration in 1 animal showed that the infection left the gland with resistance to reinfection.

Chlamydia psittaci; bovine mastitis; pathogenicity; isolation; serology; resistance.

Chlamydia, an obligate intracellular organism, includes the 2 species C. trachomatis and C. psittaci, of which the latter mainly is related to infections in mammals and birds. At the moment only 2 serotypes are considered for C. psittaci, of which a more virulent strain (serotype 2) has been isolated from the clinical syndromes of sporadic bovine encephalomyelitis (SBE) and polyarthritis in calves and lambs (*Schachter et al.* 1975). The

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other serotype (serotype 1) includes isolates from the remaining syndromes in ruminants such as epizootic bovine and enzootic ovine abortion (EBA & EAE), seminal vesiculitis syndrome (SVS) besides interstitial pneumonia and persistent intestinal infection (*Storz* 1971, *Rønsholt* 1977, *Shewen* 1980). However the abortion strains separate themselves from the rest due to a different behaviour in vivo and in vitro (*Spears & Storz* 1979, *Rønsholt* 1980).

Neither serotype 2 nor the equally virulent abortion strains seem to be prevalent among cattle in Denmark or the neighbouring countries. On the other hand the strains associated with persistent intestinal infection in cattle are more widespread (*Storz*). Thus many serologically positive reactors were reported from Denmark, and through the manure these organisms are rather common contaminants of the cattle surroundings (*Rønsholt* 1977, 1978). It was therefore studied, if this ubiquitary strain may cause other clinical syndromes than the lung and intestinal infections already observed in the calf (*Rønsholt* 1978). Mastitis would be an obvious clinical manifestation to investigate, since mastitis has already been experimentally introduced with the exotic, and more virulent abortion and serotype 2 strains (*Bannister et al.* 1959, *Enright et al.* 1958). Hence a pathogenetic and serological investigation was performed by local infection of the gland with one of the common persisting intestinal chlamydia strains.

MATERIALS AND METHODS

Animals

The investigation included 8 Jersey cows 3—5 years of age in different stages of milk yield (Table 1). The cows were fed concentrated fodder and had free access to hay. Seven of the animals received varied doses of chlamydia through the streak canal of the right front udder-quarter, which at this time appeared clinically normal and without any bacteriological, chlamydial or pathological findings of the secretion. One cow (Table 1, No. 3) received a control inoculum (see Inocula), and so did the left front quarter of the udder of the remaining animals. Once a day the cows were milked, and their production together with the clinical symptoms recorded. Milk samples were collected aseptically for daily microbiological investigations, and the appearance of the secretion graded according to the pathological

changes (Table 1). In addition to the control animal, 2 cows were sacrificed at the estimated maxima of the initial clinical symptoms, and the udder subjected to necropsy and histopathological investigation. The sampling period corresponded to that of another reported experimental infection with an abortion strain (*Corner et al.* 1968). The remaining animals were kept under observation until the clinical symptoms had disappeared.

Inocula

An egg-grown C. psittaci strain (ROS DK/KVL-6/B3), isolated from a persisting intestinal infection in a Danish cattle herd (*Rønsholt* 1978), was purified by trypsinization and differential centrifugation combined with the addition of Celite® (*Page* 1974). Finally the sediment was suspended in a phosphate buffered (pH 7.0) sucrose solution (0.2 mol/l) and stored at —80°C until use. For rechallenge trial the inoculum of an abortion strain (EBA 59-795), isolated from an American outbreak of epizootic bovine abortion (*Storz et al.* 1960), was prepared in the same manner. The control inoculum consisted of material from non-infected eggs submitted to the same purification procedure as the infectious inocula.

Isolation technique

Bacterial contamination of the samples diluted 10^{-1} and 10^{-2} were currently tested on 5 % blood-agar plates incubated aerobically and anaerobically in pyrogallol bags. For chlamydial isolation the samples were diluted 1:5 in sucrose solution and stored at 5°C for no longer than 48 h. A titration was accomplished by inoculating the yolk-sac of 5 seven-day-old embryonated eggs with 0.2 ml of a tenfold sample-dilution. The eggs were incubated at 37°C and candled daily until the death of the embryos, and smears from the infected yolk-sac, stained according to *Gimenez* (1964), confirmed the diagnosis by the presence of the organisms (*Storz* 1971, *Rønsholt* 1978). The chicken embryo 50 % lethal dosis (CELD50) was calculated by the method of *Reed & Muench* (1938).

For isolation on cell culture (L-cell monolayer), the inocula consisted of the diluted supernatant from the vortexed, centrifugated milk samples. The infection was enhanced by centrifugation and the addition of glucocorticoid and cycloheximide. The titres of inclusion forming units (IFU) were calculated on

alcohol-fixed cultures, stained by methylene blue, 48 h post infection (p.i.) (*Rønsholt* 1980). A neutralization index was calculated by incubating 10^5 IFU of the ROS strain with pre- and post infectious milk (cow No. 8) for 2 h at room temperature and 1 h at 37°C. The question of unspecific toxic influence of the milk sample was excluded by titration of agent in cell cultures pretreated with the post infectious milk.

Histological examination

Duplicate sections of the upper, middle and lower part of the inoculated mammary gland were fixed either in formalin and stained with haematoxylin-eosin, or in alcohol and stained overnight with a basic fuchsin-phenol solution as recommended by *Gimenez* (1964) but diluted 10^{-1}, followed by a prolonged counterstain for 4×1 min with intermittent wash in tapwater.

Treatment of serum

The samples were divided in two for the complement fixation test (CFT) and the indirect haemagglutination test (IHA). The former part was pretreated with 20 % guinea-pig serum complement (Oray, B.W.) and heat-inactivated ½ h at 56°C, while the latter was adsorbed with 10 % glutaraldehyde treated sheep erythrocytes for ½ h at 37°C and heat-inactivated.

Treatment of milk secretion

The samples were defatted by centrifugation ($3000 \times$ g, 10 min) and freezing at —20°C for 10 min, which made the fat-plug removable. The casein was sedimented and removed by treatment with 10 % acid v/v (HCl & CH_3COOH 1:1, 1 N) at pH 4.8 for 2 h (5°C), and the supernatant again neutralized to pH 7.1 with 1 N-NaOH. The subsequent treatment including a final two-fold dilution starting at 1:5 (Fig. 1) was similar to the procedure described for serum.

CFT

The test was performed in polystyrene microtiter U-plates with aliquots of 25 µl. Ether extracted and acetone precipitated antigen (Ag), from an EBA strain (*Storz et al.* 1960) cultivated in embryonated eggs, was employed together with 2 units of complement and overnight incubation at 5°C. More than 75 % complement fixation was considered positive (*Rønsholt* 1977).

IHA

Glutaraldehyde fixed sheep erythrocytes (*Lewis et al.* 1975) were sensitized with a deoxycholate extracted Ag from the egg-grown purificated EBA strain (*Page* 1974) by tannic acid (*Lewis et al.* 1972). A 0.8 % suspension of the sensitized erythrocytes was prepared in phosphate buffered saline pH 7.1 (PBS) with the addition of 0.1 % gelatine, 0.02 % sodium azide (PBS-GA) and 10 % dimethylsulfoxide as a freeze preservative before being stored at —80°C. The test was performed in polyvinyl microtiter V-plates with aliquots of 0.1 ml sample dilution in PBS-GA and 25 µl sensitized erythrocytes, washed once before use in the same diluter. The positive results consisting of more than 75 % agglutination were read after 3 h at room temperature, and following resuspension of erythrocytes, again after 20 h. Erythrocytes sensitized with control Ag secured the test against false positive results.

RESULTS

All glands infected with the ubiquitary intestinal chlamydia strain developed mastitis, while no pathological symptoms or isolation of organisms resulted from those inoculated with non-infected control material. In most cases the clinical symptoms started with subcutaneous oedema; there was little pain only, and heat was restricted to the infected quarter of the udder. After 3 or 4 days a palpable nodular enlargement of the gland was observed, which nearly lasted as long as the abnormal secretion persisted, while the local supramammary lymph node showed no reaction. The general condition of the test animals appeared normal, and no rise of the body temperature was recorded.

From 2 to 6 days p.i. small fibrinous clots appeared in first milk (Table 1). Usually the amount of fibrin increased somewhat, but coincidently with the end of the initial subcutaneous oedema, the fibrinous outpour subsided for a short while until a new inflammation period of about 12 days started. On an average the clinical symptoms thus stayed for about 16 days. The abnormality of secretion was characterized by milk with numerous large fibrin clots, which repeatedly plugged the streak canal. At the culmination of the inflammation the secretion had an urine-like appearance due to casein precipitation of the often defatted and blood-tinged product (Table 1: ++++). Following

Table 1. Abnormality* of milk secretion and the possibility of agent reisolation** in chlamydial bovine mastitis, induced by the ROS DK/KVL strain.

Cow No.	1	2	3***	4	5	6	7	8	8
Inocula (CELD50)	$10^{7.7}$	$10^{6.7}$	0	$10^{8.3}$	$10^{8.3}$	$10^{7.5}$	$10^{7.1}$	$10^{7.8}$	Titre of secret. (—log 10)
Init. milk yield (l)	1.5	0.5	0.5	1.0	1.5	0.2	1.0	0.2	
Final — — —	0.5	0.3	0.5	0.3	0.2	0.2	0.4	0.2	
Day post infection									
0	—	—	—	—	—	—	—	—	0
1	—	—	—	—	—	—	—	—	0.8
2	(+)	(+)	—	—	—	—	—	(+)	
3	+	—	—	++	+	—	—	(+)	
4	++	+	—	++	(+)	+	—	—	
5		+	—	+	(+)	+	—	+	
6		++	—	(+)	(+)	+	(+)	(+)	3.4
7			—	—	++	+	+	(+)	3.6
8				+	++	+	+	+	4.7
9				++	+++	++	+	++	
10				++	+++	++	+	++	
11				+++	+++	+++	++	++	
12				+++	+++	+++	++	++	3.8
13				++	+++	+++	+++	+++	3.0
14					+++	+++	+++	++	3.6
15					+++	+++	+++	+	2.7
16					+++	+++	+++	+	
17					+++	++	++	(+)	
18					+	++	+	—	
19					+	+	+	—	0.8
20					—	—	(+)	—	0.7
21						—	—	—	0.7
22							—	—▲	0.7
23							—▲	—	0
24							—	—	
25							—	—	

* Abnormality graduated according to the contents of fibrin. +++: included a urine-like appearance of the secretion, often blood-stained.

** Reisolation indicated by the negative logarithm of chicken embryo 50 % lethal dosis (CELD50). ▲: the last day of possible reisolation.

*** Cow No. 3 was only inoculated with a control inoculum, consisting of a purified egg material without the agent.

this period the secretion again turned normal except for a small number of big clots in the residual milk. In all cows but those having a preinfectious extremely small milk yield, the infection resulted in a considerable reduction of the milk production (Table 1), which appeared with the first clinical symptoms and lasted for the rest of the observation period, viz. 63 days (cow No. 7).

No bacteria could be cultivated from any of the inoculated gland-quarters during the investigation, while chlamydia was demonstrated in all infected quarters from the second day p.i. and up to 5 days after the clinical symptoms had ceased (Table 1). Following an initial reduction of the inoculated amount of chlamydia within the first 24 h, the intensity of the clinical symptoms increased and decreased following the chlamydial titre variation, as depicted for cow No. 8 (Table 1). The highest infectious titres observed per ml secretion of the various infected cows ranged from $10^{3.6}$ to 10^{5} CELD 50 or IFU. Serologically 4 of the 5 test animals seem to have experienced some kind of a chlamydial infection before. The experimental infection caused a local humoral immune response consisting of an increasing level of either agglutinating or complement fixing antibodies (Ab) around 10 days p.i. (Fig. 1). The infection was thus safely detectable by the IHA test in 3 (Nos. 8, 6 & 7) and by the CFT in 4 (Nos. 8, 6, 5 & 4) of the 5 investigated animals, while only 2 were positive in both tests.

The situation in the milk secretion seemed to be reflected in the serum; however, discrepancy existed at least in 1 cow (No. 7), which towards the end of the investigation period demonstrated an elevated complement fixing Ab level in serum alone. The CF test on serum therefore, at least periodically, was able to detect the infection in all animals, though a safe diagnosis at any arbitrarily chosen point required supplementary information from the IHA test. For the other cases the serum reaction was sometimes seen to dominate quantitatively and to be the first demonstrable immune response, especially if the diluting effect of respectively the blood and milk volumen was taken into account.

The infected gland of cow No. 7 (Table 1), which had recovered, was rechallenged through the teat canal with $10^{8.2}$ CELD50 of the ROS strain on day 26 p.i., but neither clinical symptoms nor reisolation of agents resulted from this inocu-

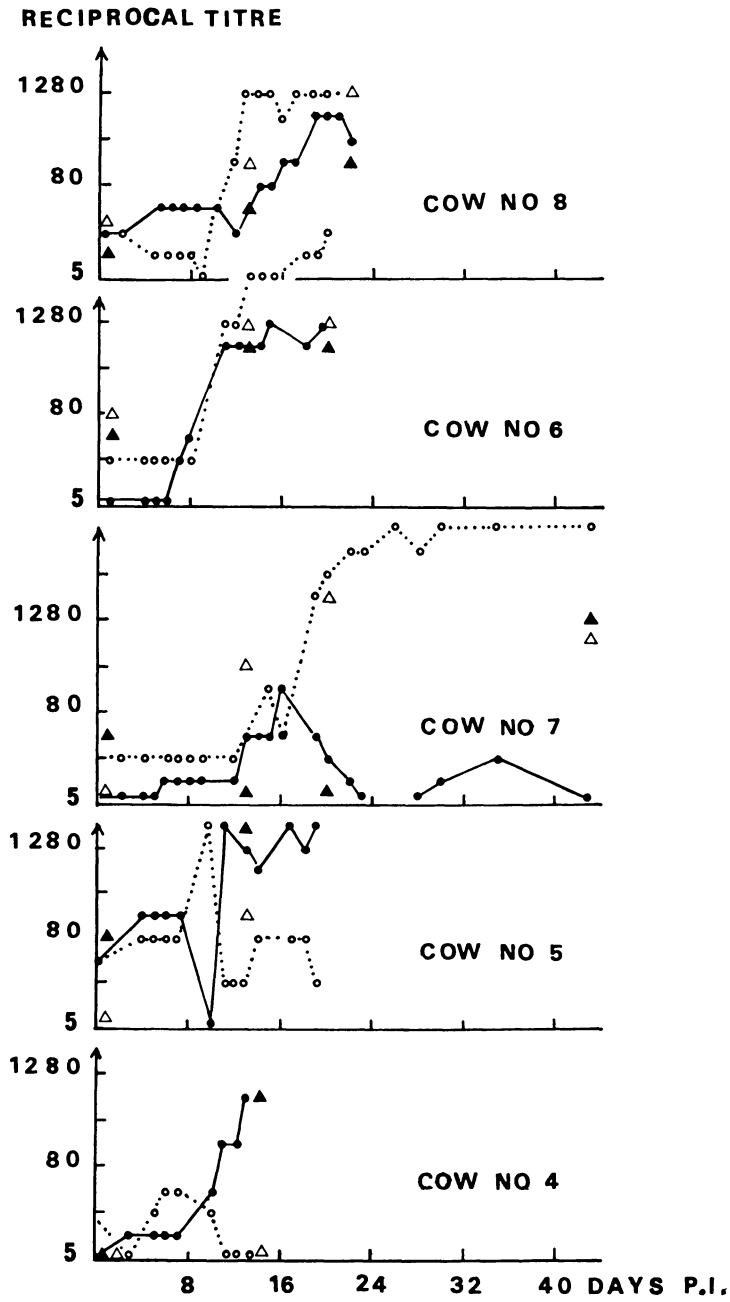

Figure 1. Experimental chlamydial bovine mastitis days post infection (p.i.). Agglutinating antibodies in whey (○·····○) and serum (△). Complement fixing antibodies in whey (●——●) and serum (▲).

lation. A new challenge trial took place 50 days after the first inoculation with $10^{8.4}$ CELD50 of the EBA strain, and this only gave rise to fine fibrin clots restricted to the day following inoculation. Beyond this no clinical symptoms appeared, and reisolation of the agent was not possible. Milk samples from cow No. 8 with a similar clinical course as that of the challenged animal were used in a neutralization test employing the ROS strain and cell culture technique. The tests were accomplished with samples collected in the period corresponding to the challenge time viz. 28 and 34 days p.i. and stored at —20°C for several weeks until use, which resulted in an increased neutralization index in cell culture of 125 and 250, respectively, compared to 7 in embryonated eggs 34 days p.i.

At post-mortem examination, the udder of the 2 infected cows sacrificed 4 and 6 days p.i. showed interstitial oedema and lobular consolidated areas with fibrinous exudation. Especially the lower part of the gland was affected. The mucous membrane of the cistern demonstrated disseminated ulcerations. The histological investigation of the affected areas revealed an acute lobular exudative mastitis, dominated by a mononuclear cell

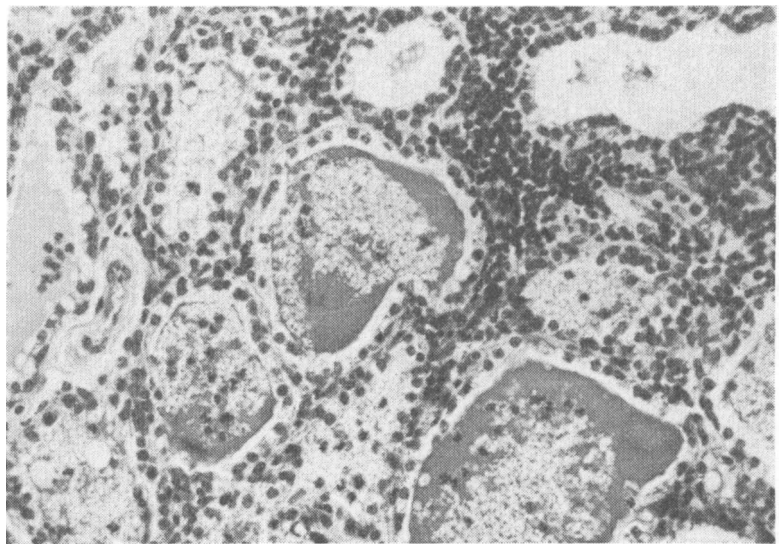

Figure 2. Part of a lobule from an udder experimentally infected with Chlamydia psittaci, demonstrating exudative alveolitis with a few neutrophilic leucocytes and a heavy interstitial, mononuclear cell infiltration. H & E, × 250.

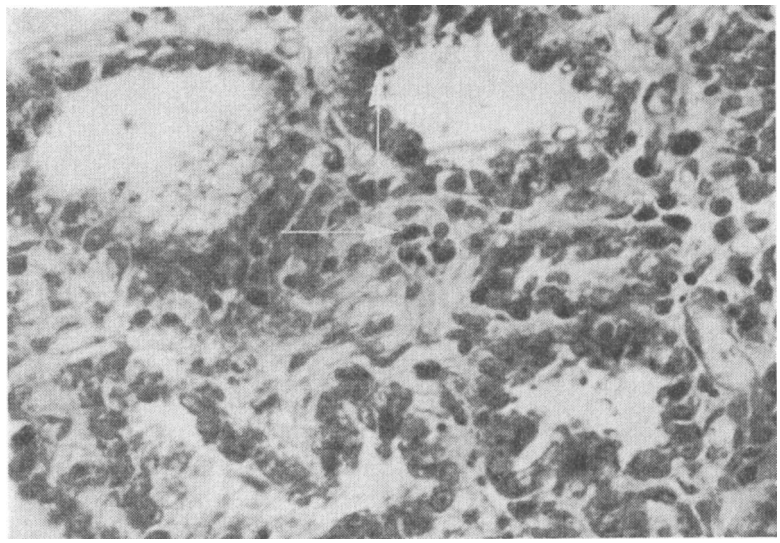

Figure 3. Part of a lobule from the periphery of a less inflamed area (Fig. 2). Chlamydial inclusions (arrows) are seen in many epithelial cells and in macrophages infiltrating the interstitium. Modified Gimenez, × 535.

infiltration of the interstitial and septal tissues. Many capillaries had perivascular cell infiltration and swollen endothelial cells. The alveoles and ducts were plugged with fibrinous outpoor, neutrophilic leucocytes and desquamated epithelial cells, focally stripping the basal membrane (Fig. 2). While the architecture was hard to recognize due to the inflammatory disorganization of some of the lobules, chlamydial inclusions could be demonstrated by the modified Gimenez stain in alveolar and ductal epithelial cells belonging to the less affected areas in the upper part of the gland, and in some of the mononuclear cells of the septa (Fig. 3). Sections from the cisternal wall displayed disseminated necrosis of associated gland tissue and epithelium besides epithelial metaplasia. Sections from the gland of the control inoculated cow showed no signs of pathological changes nor any inclusions by the special stain.

DISCUSSION

The pathogenetic potential of the ubiquitary intestinal chlamydia strain (ROS) in causing mastitis was demonstrated. The agents invaded the gland epithelium, in which the inclusions

were demonstrated by a modified Gimenez stain. The nature of the symptoms induced by the ROS strain in the present work was identical with the mastitis produced by the more virulent strains of serotype 2 (SBE) and of abortion (EAE) (*Enright et al.* 1958, *Corner et al.* 1968), though the course and strength of the infection differed in some respects. Thus the ROS strain produced a self limiting exudative and ascending mastitis with a fibrinous to urine-like secretion, which resulted in a reduced milk production due to atrophy of the gland tissue. Cows with an initial small milk yield (Table 1, Nos. 6 & 8), however, most probably were able to compensate for this reduction by the activation of gland tissue. Contrary to the more virulent strains (*Corner et al.* 1968), the ROS strain caused a clinically more restricted, local infection with less pronounced oedema, necrosis and trombosis of vessels. The related lymph nodes showed no macroscopical or histological symptoms of agent propagation. However, there was an indirect quantitative serological evidence (Fig. 1), that the agent may have reached this far or to other lymphoid organs outside the milk gland. This indicates, that the streak canal possibly could serve as the entrance route of a systemic infection or at least as a way of obtaining an universal serological reaction against the topical agent, as it was previously demonstrated with the more virulent abortion strain (*Boulanger & Bannister* 1959). Minor secretion abnormalities often initiated the main series of clinical symptoms, but the incubation time was in general longer and the number of noxious agents lower, leading to a prolonged mastitis period compared with that of the more virulent strains (*Bannister et al.* 1959, *Corner et al.* 1968).

From the present results (Fig. 1) it is difficult to know, which immunoglobulin (Ig) classes contribute to the local immune response in the two tests employed. However, according to the findings in other reports (*Norcross* 1971, *Guidry et al.* 1980), and the time at which the different Ig groups appear (*Ernø & Aalund* 1972), the response is most probably mainly related to the immunoglobulins A & G_1, of which the former generally forms the agglutinating, and the latter the complement fixing Ab (*Tizard* 1977). The most complete immune response is represented in 2 cows (Nos. 8 & 6) within the dry-milk period, while the agglutinating Ab is quickly suppressed in 2 other (Nos. 5 & 4) with some milk production. The higher flushing

rate of the milk and the production stage of the udder would be an obvious explanation for the declining locally produced Ig. However, since another cow (No. 7) with a similar milk production responded with a high agglutination titre, the results may be attributed to individual variation among the animals. Thus it is known, that Ig M sometimes is the only class presented in the response of cattle (*Tizard* 1977).

It follows from the rather small number of investigated cows, that the 2 serological methods respectively are not sufficient for a correct diagnosis. The CF and the IHA tests have to supplement each other in order to detect an acute infection, especially when dealing with local secretions, however an indirect antiglobulin test may cover the results of both. The time, at which the immune response started, was somewhat related to the individual development of the clinical symptoms and thus to the agent propagation. On the other hand, the 2 kinds of induced antibodies demonstrated, seemed to have an equal influence on the termination of the infection. An induced resistance was demonstrated in 1 animal (No. 7) by means of a relative high rechallenge dose of different chlamydia strains. Judged from in vitro neutralisation tests in cell culture and embryonated eggs with milk secretion from an identical post infection period of another cow (No. 8), this increasing resistance mainly seems to be influenced by Ab, since the reaction was obtained with samples stored for weeks at —20°C. The different efficiency of the neutralizing Ab demonstrated in the two test systems is not explained. However, this phenomenon may partly be the reason for the improved chlamydial isolation results sometimes seen in embryonated eggs (*Rønsholt* 1980).

Although the virulent strains induced a mastitis with more violent clinical symptoms than those of the ROS strain (*Enright et al.* 1958, *Corner et al.* 1968), the final effect of this strain was even more injurious to the gland, as the infection stayed for a longer time and apparently caused a greater persistent reduction of the milk yield. Besides, the pathogenic capability of the intestinal chlamydia strains in producing mastitis is far more important because of its ubiquitary status (*Storz* 1971, *Rønsholt* 1977, 1978). However, it is questionable if the same common intestinal infection under field conditions does not partly render the udder resistant due to transfer of the relevant Ig A producing plasma cells from the sensitized intestine (*Norcross* 1971, *Husband & Watson* 1978).

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SAMMENDRAG

Bovin mastitis induceret med den almindeligt forekommende intestinale Chlamydia psittaci stamme. — En patogenetisk og serologisk undersøgelse.

Kvæg er hyppigt inficeret med en persisterende intestinal C. psittaci stamme, som gennem gødningsafsættelsen vil inficere omgivelserne. Muligheden for at en sådan stamme kan fremkalde mastitis blev undersøgt ved at inokulere 7 køer lokalt i en yverfjerdedel gennem pattekanalen, hvorefter sygdomsforløbet blev sammenlignet med det, der kan fremkaldes af mere virulente stammer (EAE & SBE).

Agens forårsagede en lokal, selvbegrænsende eksudativ mastitis med en fibrinøs sekretion, der førte til en permanent tilbagegang i mælkeproduktionen.

Stigende titerværdier af komplementbindende- og agglutinerende antistoffer blev demonstreret i valle og i serum. De to serologiske metoder gav ikke identiske resultater, hvorfor begge findes nødvendige for i alle tilfælde at kunne demonstrere agens' tilstedeværelse lokalt. Resultaterne fra èt dyr viste endvidere, at den overståede infektion gav anledning til beskyttelse imod reinfektion.

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Reprints may be requested from: L. Rønsholt, the Department of Veterinary Virology and Immunology, Royal Veterinary and Agricultural University, Bülowsvej 13, DK-1870 Copenhagen V, Denmark.