

Epidemiology and Pathogenesis of the *Bovine herpesvirus 2* Infection.

Disease in Response to Initial Antibody Production.

Abstract

The aim of the author was to present conclusions from the results of own examinations and results from literature that will lead to a new and better understanding of the epidemiology and pathogenesis of the *Bovine herpesvirus 2* (BoHV-2) infection.

The current literature on the BoHV-2 infection was reviewed, and it was concluded that important questions concerning the pathogenesis and the routes of transmission within and between herds were still not clarified. It was especially noted that the sensitivity of the tests used for the demonstration of antibodies had generally been very low.

The infection was first seen in Denmark as ulcerative mammillitis (bovine herpes mammillitis, BHM) in September 1980, and up to December 1983, a total of 53 outbreaks were diagnosed. No outbreaks have been confirmed later. The Danish test for neutralizing antibodies was elaborated to show optimal sensitivity. A herd tested shortly after an outbreak showed that all tested animals, including bull calves up to the age of 14 months, were antibody-positive. All 34 outbreaks in 1980 and 1981 apart from the last one occurred in a limited geographical area, where semen used for artificial insemination (AI) usually came from 2 local AI centers. Some selected bulls from these centers were tested and found antibody-positive.

From the Danish examinations, it was concluded, 1) that cows showing clinical BHM would be antibody-positive when tested by the highly sensitive Danish test, 2) that spread in herds was most likely to be airborne, and 3) that the first spread to herds had most probably occurred from AI centers.

BoHV-2 is also the cause of the rare pseudo-lumpy skin disease (PLSD), where skin lesions all over the body predominate. PLSD has often been reproduced by intravenous virus inoculations. From the results reported in the literature, it is further concluded (4) that experimental PLSD skin lesions appearing after intravenous inoculation were likely to have been a delayed, adaptive, universal response of the animals to the virus antigen. As a consequence of this, the inflammatory reactions leading to these lesions were the result of complement activation by the classical pathway, which implies that the pathological changes observed clinically were triggered by specific antibodies to the virus at sites of virus propagation. An identical reaction causing the inflammatory BHM lesions in the skin of teats and udders of cows would explain why affected animals are already antibody-positive when they show clinical symptoms of BHM.

Udder edema aggravates BHM disease, most probably because of reduced blood circulation in affected tissue areas, which will hamper the removal of cell-toxic substances formed during the complement activation process. Cold weather causing lowered skin temperature, in particular in hairless areas, during the critical short period after the production of a sufficient level of reacting antibodies might likewise be a disease determinant. This may explain why BHM in Europe has predominantly occurred in cold seasons. In cases of BHM, where lesions have appeared only on the

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teats, a traumatic effect of mechanical milking may have contributed to the development of the clinical picture.

Introduction

The intentions of the author

The aim was to present conclusions from own investigations and from results in papers by other authors that will lead to a different and better understanding of the epidemiology and pathogenesis of the *Bovine herpesvirus 2* (BoHV-2) infection.

Clinical pictures

BoHV-2 was first isolated in South Africa by Alexander et al. [1] from cattle showing a mild clinical disease similar to lumpy skin disease, which however is caused by the *Lumpy skin disease virus*, a capripoxvirus.

In 1964, Martin et al. [2], described an apparently new disease condition in cattle named ulcerative mammillitis. The disease occurred in the autumn of 1963 in several dairy herds in the west of Scotland.

A virus isolate was found to be a herpesvirus serologically indistinguishable from the South African isolate [3]. Already 1960, however, Huygelen [4] reported the isolation of a virus from cattle in central Africa with skin lesions on the teats and udder, which was found to be serologically identical to the South African virus.

Other authors have later confirmed that viruses from cases of pseudo-lumpy skin disease (PLSD) and bovine herpes mammillitis (BHM) are serologically identical [5,6,7], and the two last mentioned author teams were not able to demonstrate a difference in pathogenicity either after experimental intravenous infection of cattle with viruses from clinically different outbreaks.

PLSD is characterized by the appearance of multiple edematous nodules in the skin, particularly in the neck, back, head, chest, and tail region [8,9,10,11]. The nodules are generally 0.5 to 2 centimeters wide and painful on palpation. Very often, the superficial skin layer over a nodule becomes necrotic with serous exudation followed by scab formation. Experimentally, this PLSD picture has regularly been reproduced by intravenous injection of virus from both PLSD and BHM cases without any significant differences [12,13,14,6,7,9].

BHM differs clinically from PLSD in the way that skin lesions practically always are limited to the teats and udders of lactating cows. Typically, cows have lesions on one or two teats [3]. Severe lesions are generally of sudden onset, with very painful edematous swelling of the teat, discoloration, serous exudation, and sloughing of the affected skin, which leaves a distinct, deep ulcer several centimeters wide. Milder cases show swollen plaques, less exudation, and superficial ulcers. Also, udder lesions are relatively often seen, and Deas and Johnston [15] pointed out that cows and heifers with pronounced post-parturient udder edema were most likely to develop udder lesions. Large parts of the udder could be involved, resulting in severe serous exudation and very often sloughing of necrotic superficial parts of the skin.

Although in one BHM outbreak, several cows with teat and udder lesions also showed skin lesions on the head, neck, and shoulders [16] and in another one, a single case of PLSD was seen [17], the overall picture is that PLSD and BHM are two clinically distinct disease entities resulting from the BoHV-2 infection.

Occurrence

Clinical outbreaks caused by BoHV-2 have been confirmed by virological examinations in many countries (USA [8], Italy [18], Australia [19], Switzerland [20], Ireland [21], Canada [22], and Israel [11]). Only a very few outbreaks of PLSD have been recorded. In the United Kingdom, the many outbreaks reported have appeared as BHM, although a few individual cases of PLSD have been seen [16,17,10]. The first one observed in the USA was a PLSD outbreak [8], but BHM outbreaks were encountered later [23].

A few limited serological surveys for antibodies to BoHV-2 have been performed, which have evidenced a rather widespread occurrence of the infection in some countries (USA [24], Australia [25],

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Switzerland [26], and the Netherlands [27]. In East Africa, Plowright and Jessett [28] isolated the virus from a buffalo, and testing of sera from cattle and wild ruminants demonstrated that the infection was widely distributed in both cattle and buffaloes, but also to some extent in other wild ruminants.

Transmission of the infection

How the infection is spread within herds or between herds is still uncertain as judged from literature. Martin et al. [2] reproduced the clinical disease by inoculating the virus isolated into the teat skin of two cows to fulfill Koch's postulate regarding the demonstration of a causal relationship between the agent and the disease. Since the disease was restricted to lactating cows, and since teats seemed to have become infected, because the virus had entered the teat skin from outside, Martin et al. [3] concluded that infection was most probably transmitted within a herd by the milking machine or the milkers' hands. Pepper et al. [29] observed the disease in 18 herds in a restricted geographical area in the south-west of England and suggested that biting flies might play a role in the spread of the infection. Rweyemamu et al. [13] accepted these two points of view as being the only likely possibilities of spread within herds.

Gibbs and Collings [30] observed a few outbreaks, where heavily pregnant heifers showed BHM lesions before or within one or two days after calving, and subsequent experimental studies on infection of teat skin demonstrated that intact skin was very resistant to infection [31]. It was therefore concluded that the milking machine seemed to be of little importance in the spread of the infection. It was also found that the virus had to be injected deep into the skin to reproduce lesions similar to natural ones, and the possibility of transmission by *Stomoxys calcitrans*, which was thought to be a likely vector, was investigated. The result was inconclusive, but nevertheless, it was deduced that natural infection was likely to be regularly transmitted by insect vectors within and between herds.

Ways of transmission are still not clarified. In recent papers, Kemp et al. [32] maintain that the epidemiology is uncertain, and Brenner et al. (2009) [11] mention that the infection is considered to be transmitted by biting flies.

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The BoHV-2 infection in Denmark

The virus-neutralizing antibody (VNA) test

Bitsch [32] studied the antigen-antibody reaction in serological tests as measured by the neutralization reaction and demonstrated regular lines for the course of the reaction, based on which highly sensitive antibody tests could be elaborated. The reaction was temperature-dependent and furthermore with extended reaction strictly proportional to the length of the reaction period. Consequently, an increase of the reaction period for virus-serum mixtures before inoculation of tissue cultures by a factor of e.g. 16 (4 in $\log_2$) should increase titers, and therefore also the test sensitivity, by the same factor.

In studies with VNA tests for *Suid herpesvirus 1* [34] and *Bovine herpesvirus 1* [35], incubation of virus-serum mixtures at 37 °C for 24 hours instead of 1 hour increased the sensitivity of these tests by 4.1 to 4.2 in $\log_2$, and the small deviation from $\log_2 24 = 4.59$ was due to a regular over-neutralization phenomenon seen with very short reaction periods and still observable to some degree after 1 hour of reaction.

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As BoHV-2 showed no thermal inactivation at 37 °C for at least 24 hours, the Danish routine test for virus-neutralizing antibody was performed with incubation of virus-serum mixtures at 37 °C overnight, i.e. for 19-22 hours. Serum samples were tested undiluted, and positive samples were titrated. More than 1000 blood samples received for other examinations, mainly in connection with the export of cattle, had been tested to document acceptable specificity, and all were clearly negative, demonstrating that the test specificity, despite the high sensitivity, was extremely high.

Outbreaks of BHM

In November 1980, material from a teat lesion and a blood sample were received from a cow in Jutland with clinical symptoms of BHM. BHM virus was isolated from the lesion material and antibody was demonstrated in the blood sample. One week later similar material was received from another veterinary practice. The virus was isolated and again antibodies could be demonstrated in the serum of the affected animal. Up to November 1983, material was received from a total of 53 herds with clinical symptoms of BHM. In most of these cases, only blood samples were received, but because of the extremely low prevalence of the infection and the strict correlation between the clinical picture and

the presence of antibodies in the blood sample, it was accepted that a blood sample from an affected cow would be sufficient for confirmation of the clinical diagnosis. In no case was a blood sample received for confirmation of clinical BHM that did not turn out to be positive. All positive blood samples were titrated, and the titers were low, generally between 4 and 32 (2 and 5 in log₂), which means that a very great proportion of these samples would have been found negative if a reaction time of 1 hour had been used (see later). BoHV-2 virus was isolated from a total of 6 herds.

Table 1.
Occurrence of 53 outbreaks in Denmark of bovine herpes mammillitis 1980-1983 by the month of the year.

	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sep.	Oct.	Nov.	Dec.	Total	No. of veterinary practices involved *)
1980									1	5	1	9	18	6
1981			3	4		2	1	2	1	3	2		18	6 (4)
1982					1		1	1		4	1		8	6 (3)
1983		2						5	3	1			11	8 (5)
Total		2	3	4	1	2	2	8	5	13	4	9	53	18

*) In brackets: new veterinary practices

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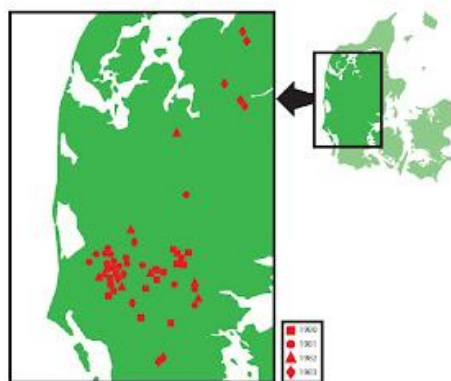
The geographical location of all 53 herds with outbreaks is shown in Figure 1, and in Table 1 these same outbreaks are given by the month of occurrence. The special seasonal appearance seen in the UK with practically all BHM outbreaks occurring in the last six months of the year [36, 37], was not observed in Denmark, although 41 of the 53 outbreaks (77 %) did occur in that part of the year.

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It is remarkable that from November 1983 up to January 1986, when this author left the National Veterinary Diagnostic and Research Laboratory, no further outbreaks of BHM were registered. In 2004, in connection with a study on viral bovine teat lesions, questionnaires were forwarded to Danish veterinary practices. In none of the 27 practices responding had BHM been observed for many years, and nor had the infection been confirmed at diagnostic laboratories after 1983 (S. Andersen and T. Skovgaard, personal communications).

Figure 1.

Geographical location of 53 outbreaks of bovine herpes mammillitis in Denmark 1980-1983.



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Additional examinations

A few weeks after the first outbreaks, blood samples from one of the herds with approx. 35 milking cows were tested for antibodies to BoHV-2. The only animals not tested were heifers 1-2 years of

age, being pastured day and night. All animals, among which were own bull calves being fattened for slaughter at the age of approx. 14 months, were antibody-positive.

All 34 outbreaks in 1980 and 1981, apart from the last one in October 1981, occurred in an area, where semen used for artificial inseminations (AI) usually came from two AI bull centers. On one occasion in 1981, (1) samples from all 30 waiting bulls from one of these centers separated from the rest of the bulls, (2) a sample from a bull at the other AI center in the area, and (3) samples from all bulls at a third AI center, all received for other examinations, were subjected to an unauthorized testing for BHM antibodies. The only negative samples were those from the bulls at the third center.

Conclusions

Blood samples from the clinically affected cows forwarded for laboratory confirmation were invariably positive when tested by a VNA test with optimal sensitivity, although with relatively low titers. The strict correlation between clinical symptoms and the presence of antibodies led to the conclusion that symptoms were most likely related to the appearance of antibodies.

The results of the serological examination of all animals apart from pastured heifers in one of the first herds infected, where all animals, also bull calves up to the age of 14 months, and the examination of all 30 AI waiting bulls from one center, illustrated that this infection was easily transmitted to all near-by cattle. No explanation of this feature other than airborne spread by the respiratory route was conceivable.

The BoHV-2 infection should, therefore, ordinarily be a transient or asymptomatic respiratory infection of cattle where the acute infection period in affected animals would be over when clinical disease with skin lesions appears.

The BoHV-2 outbreaks appeared suddenly in the autumn of 1980 in several dairy herds in Mid-Jutland. The infection had never before been diagnosed in this country and was most probably the result of a recent introduction, but the immediate explanation of a transmission between these herds could be seen. In this respect it is notable (1) that the first 33 outbreaks in 1980 and 1981 occurred in an area, where the semen commonly used for AI originated from two local bull centers, and (2) that infected animals were found at these centers. Logically, therefore, semen from the centers contaminated with the virus was the most likely source of infection.

Discussion and conclusions

Inflammatory disease in response to the formation of humoral antibodies

Several research teams infected cattle by the intravenous route and were able to reproduce the special PLSD picture characterized by multiple skin lesions. Haig [12] inoculated 2 calves, and numerous skin nodules developed on the eighth day on their necks and later also on their heads and backs. Rweyemamu et al. [13] inoculated 7 animals intravenously, but reactions were seen only in a calf and a cow, which reacted by numerous cutaneous plaques, mainly on the neck and head. In the calf, these skin lesions were seen after 7 days, but in the cow, the first skin nodules were seen on the neck after only 3 days, but by the eighth, they had increased in size and number. Martin et al. [38] inoculated 2 cows intravenously. They reacted by the formation of skin lesions after 7 and 8 days. One cow had numerous skin lesions, while the other one only had one on each side of the neck. Kalunda and Plowright [14] obtained multiple cutaneous lesions in 4 of 10 animals inoculated with relatively high virus doses, which appeared after 6 to 8 days. Castrucci et al. [18] infected 2 calves intravenously, which responded by disseminated skin lesions after 6 to 7 days. Castrucci et al. [6] inoculated a total of 6 calves intravenously with 3 different BoHV-2 strains, using 2 calves per virus strain. After 4 days small necrotic lesions could be seen in the nose close to the nostrils, and virus was isolated from nasal swabs from all calves. One week after inoculation, cutaneous lesions could be seen on various parts of the body in all 6 calves, although with differences in number and location. In a subsequent study [39] comprising 6 calves, generalized lesions developed in all animals 6 days after inoculation. (8) Gigstad and Stone [7] inoculated 6 animals using three different virus strains, and cutaneous lesions were seen 6 days later in 4 of them, but after 5 days in the remaining two, which had been kept together with a naturally seropositive animal. StGeorge et al. [9] reported that 2 calves after intravenous inoculation developed skin lesions on the sixth day, almost all over the animals, most easily seen in the head, neck, and back regions.

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Other routes of inoculation have also resulted in skin reactions in rare cases. Gibbs and Collings [30] injected the virus intradermally into 2 calves and had skin nodules in both after 6 days, and Castrucci et al. [18] inoculated 2 calves nasally and saw skin reactions after 9 days in one of them.

High doses of the virus were typically administered to these experimental animals, which therefore cannot immediately be compared to natural cases. Nevertheless, a general picture seems to arise from the many cases reported, namely that all of a sudden, usually 6 to 8 days after the intravenous inoculation, numerous skin nodules develop; and where uniform groups of animals are inoculated with identical virus doses, the animals tend to show the skin nodules at practically the same time. Such a response can hardly be considered an immediate, simple reaction to a gradually progressing growth of virus, but must be explained by a delayed, adapted ability of the host to react to the presence and activity of the virus.

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This obvious connection between the observed disease symptoms and an adaptive host response does not seem to have been realized earlier. Neutralizing antibody to BoHV-2 has been demonstrated from 9 to 10 days after experimental infection by tests of varying low sensitivity [14, 40], so the sudden appearance of multiple skin nodules approximately 6 to 8 days after intravenous inoculation of high virus doses would seem to be in reasonable accordance with the time of initial antibody production.

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Virus in high concentrations has been demonstrated in such skin lesions, and histological examinations, also of teat lesions, have demonstrated intensive inflammatory reactions with dilation of capillaries, marked edema, cellular infiltrations, mainly of lymphocytes, and later also the formation of necrotic tissue [41, 42, 38, 39, 40].

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Inflammation is caused by complement activation, cf. Markiewski and Lambris [43]. Complement is a series of enzymes, which on activation starts a cascade of reactions aiming at destroying invading viruses, bacteria, etc., and infected cells. Briefly, activation of complement gives rise to the release of mediators, which locally will cause inflammation characterized by capillary dilation and vascular permeability leading to edema, exudation, emigration of leukocytes, and reduced blood circulation. This again may also result in adverse severe tissue injury. And affected tissue areas will even very early in the inflammatory process be painful because of the release of mediators involved in the pain reaction.

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One pathway of complement activation is *the classical pathway*, where the complement is activated by the formation of antigen-antibody complexes. Activation by this pathway will be delayed as related to the time of infection because it will not take place until specific antibodies have been produced, while activation by the other pathways does not have to await a similar time-dependent response from the host. Complexes of the virus formed with specific IgM and IgG antibodies will activate complement, which implies that activation (1) will occur as soon as a sufficient IgM and/or IgG antibody level to the virus has been produced and circulated, and (2) will occur specifically at the sites, where the virus has been propagated.

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In conclusion, the facts (1) that cows in Denmark with acute BHM were invariably found antibody--positive by a sufficiently sensitive VNA test, and (2) that the sudden appearance of multiple skin lesions in PLSD cases 6 to 8 days after experimental inoculation, are explainable if the skin lesions result from an adaptive host response to the infection. Furthermore, the skin lesions found in cases of experimental PLSD or natural BHM show the typical inflammation reactions caused by complement activation, which lends strong support to the notion that skin lesions seen in BoHV-2 infections are caused by complement activation by the classical pathway. This involves that reactions will take place locally at the sites of virus propagation when the early specific antibodies have been bound to the virus.

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How does the association of disease with the initial antibody formation correlate with epidemiological data?

In papers mentioned above, it was concluded that cattle most probably got infected by the introduction of the virus through the skin. This first led to the idea that infection was transmitted by the cups of the milking machine, and later to another explanation that biting insects were vectors.

BoHV-2 has been termed a *dermatotropic* virus because of its involvement in skin reactions, and PLSD has in contrast to BHM been considered a manifestation of a generalized infection. But the virus might very well in cases of BHM have reached the skin of a teat by the bloodstream. For example, PLSD has been seen occasionally in natural outbreaks of BHM [16, 17], and also in a case of experimental nasal infection. Here, viremia was demonstrated in another nasally infected calf [18].

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Martin et al. [38] and Gibbs et al. [31] found experimentally that fluid from teat lesions had high virus titers, but this does not seem to be concordant with findings in infected herds. Rweyemamu and Johnson [44] pointed out that isolation of the virus from teat lesions was difficult, unless samples were taken from very early lesions, and Pepper et al. [29] investigating material from 18 outbreaks succeeded in isolating the virus from only one sample. This disagreement might be due to the appearance in natural cases of antibodies before teat lesions, because of which recovery of the virus would be impeded.

Also, the use of antibody tests of inadequate sensitivity has given rise to confusion. For example, Martin et al. [3] and Kalunda and Plowright [14] found experimentally infected animals antibody-negative. Janett et al. [45] used a more sensitive test modification, which however was still approximately 11 times (3.5 in $\log_2$) less sensitive than the Danish test. They followed a dairy herd after an outbreak of BHM by serological examinations on 7 occasions within 15 months. Of 21 animals, 10 were antibody-negative and 2 were antibody-positive every time, while 9 animals gave varying results. In this last group were 4 cows that had shown clinical symptoms. Inadequate serological tests will have led to false antibody-negative results, for example when experimental in-contact animals were found negative, or when animals in naturally infected herds were tested. Even falsely seronegative animals may have been selected for experimental examinations.

The likelihood of a spread of the infection by the respiratory route has neither been substantiated nor refuted in literature. But examinations have been limited. Castrucci et al. [46] infected 7 calves intravenously or intradermally, and all shed the virus nasally 7 and 10 days later. When they were treated with dexamethasone 6 months later, nasal swabs from all were again virus-positive. Also, experimental nasal infections have been successful [18], and Rweyemamu et al. [13] found seroconversion in a control calf in a study, where animals were inoculated intravenously. In this respect, it is also worth noticing that results from several examinations were found controversial, only because the possibility of respiratory spread was not considered [14, 27, 9, 47, 45, 32].

Udder lesions have been seen especially in recently calved heifers and cows with udder edema, while cows later in lactation predominantly have shown teat lesions. Lesions have appeared on teats and udders of heifers as early as 1 to 3 days after parturition, or even before [30], and Martin et al. [37] considered such cases as possibly earlier infected animals, where a latent infection had been reactivated by stress during calving. But animals showing BHM lesions around parturition may have gotten infected a few days earlier in the same way as non-lactating animals in the herd.

When thoroughly investigated, the virus has been found present at the sites of udder and teat lesions, and the inflammatory reactions seen here might be explained by complement activation by the classical pathway. Edematous udder skin will have reduced blood circulation and a lowered temperature. An inflammatory reaction caused by complement activation in such skin must, therefore, be expected to be correspondingly strong, because the nutritional supply to cells and removal of tissue-damaging mediators or toxins will be impaired. Tissue injury alone will also increase complement activation. This may be part of the explanation for why extensive superficial necrosis of udder skin is often seen in heifers or cows with udder edema.

Udder edema appears to aggravate BHM disease, and cold weather might, in the same way, be an additional determinant of disease because of a lowered skin temperature and subsequently reduced blood circulation. BHM is most often seen in lactating cows in relatively cold periods when many of these animals are still pastured during the daytime. Viremia may very well be a regular feature of a natural BoHV-2 infection and the virus will because of a particular affinity where in most cases local non-excessive inflammatory reactions in the skin with sufficient circulation of blood might proceed uncomplicated. The fact that delayed skin reactions after intravenous inoculations have appeared especially when large doses of virus were used might be related to an excessive immune response in such cases.

It must be realized also that the milking machine in some cases may have an aggravating effect on tissue lightly damaged by the inflammatory processes.

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In summary, the results and observations from the occurrence of BoHV-2 infection in Denmark were (1) that cows showing clinical BHM were invariably antibody-positive when examined by a very sensitive test, (2) that spread in herds was most probably airborne, and (3) that the first spread to herds might very well have occurred from AI centers. It was furthermore concluded (4) that natural teat and udder lesions – just as the experimental multiple skin lesions appearing suddenly 6-8 days after intravenous inoculation – will have to be the result of an adaptive universal response of the animal to the virus antigen, implying that the inflammatory reactions leading to these lesions have resulted from complement activation by the classical pathway initiated by the early formation of specific antibodies to BoHV-2.

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It seems to be the first time that the adaptive immune response has been incriminated as the cause of illness in cattle. Lumpy skin disease may be another similar disease, and more may be recognized in the future.

Even from the knowledge concerning the BoHV-2 infection generally accepted today, one must conclude that BoHV-2-infected animals are undesirable at AI centers. A test used for control before admission must not be less sensitive than the Danish VNA test modification described here.

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Comment [A23]: without three refernces which is published in ,2000,2007 and 2009 the rest it is too old there are alot of books currently like radostata, medndel, etc. for disereses try to refer update

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