Control of Bluetongue Serotype 8 in Europe

Proceedings of a Symposium Presented at the XXVth Jubilee World Buiatrics Congress

Intervet
Schering-Plough Animal Health

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Bluetongue:
The Virus and the Infection in Cattle and Small Ruminants
Etienne Thiry, Professor, DVM, MS, PhD, DECVPH

Midges As Vectors of Viruses of Bluetongue Disease and Measurements of Protection
Heinz Mehlhorn, Professor, PhD

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Intervet
Schering-Plough Animal Health

Preface

Welcome to the symposium "Control of Bluetongue Serotype 8 in Europe"!

As the leading manufacturer of vaccines for animals worldwide, Intervet/Schering-Plough Animal Health is pleased to sponsor this symposium at the XXVth Jubilee World Buiatrics Congress in Budapest, Hungary. Our comprehensive program covers the most important aspects of bluetongue disease, including the best approaches to disease control through the use of insecticides and vaccines.

Major changes have occurred in the epidemiological situation of bluetongue infections in Europe over the past several years. Until 2006, some Mediterranean countries had been affected by serotypes 1, 2, 4, 9, 16, or a combination. Summer of 2006 proved to be a turning point, however, with the unexpected appearance of serotype 8 in northern Europe. Countries in that region were traditionally considered unlikely to be affected by bluetongue disease. After the first outbreaks of BTV serotype 8 in The Netherlands, the disease quickly spread to other European countries. Infections reached alarming levels during 2007, and without appropriate control measures further spread is expected to happen in 2008.

Previous experience with bluetongue virus serotypes has shown that only adequate control measures can stop the disease from becoming endemic in Europe. Vector control with insecticide treatment, such as deltamethrin (Butox® 7.5 pour-on), and hygienic measures must be combined with (mass) vaccination programs. In this proceedings Prof. Etienne Thiry and associates at the University of Liège, Belgium, give an overview of bluetongue virus and the resulting infection in cattle and small ruminants. Prof. Heinz Mehlhorn of Heinrich Heine University in Düsseldorf, Germany, describes midges as the bluetongue vectors as well as measurements of protection by treatment with insecticides. Finally, Dr. Birgit Makoschey and associates in Boxmeer, The Netherlands, and Barcelona, Spain, detail the experimental evaluation of an inactivated vaccine that has proven safe and effective against serotype 8.

Intervet/Schering-Plough Animal Health maintains a strong focus on research and development, which is evident in its constant introduction of innovative products that meet specific market needs. Immediate response to detection of bluetongue virus serotype 8 enabled the R&D team to develop an effective inactivated vaccine quickly so that vaccination of animals against the virus will be possible in 2008.

I trust you will find the symposium informative and the content of this proceedings useful. Intervet/Schering-Plough Animal Health is highly committed to winning the battle against bluetongue disease. We are always open to comments on our work, products, and presentations. Feel free to contact us at www.intervet.com to express your concerns and practice needs.

Dr. René M. L. Aerts
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About the Authors

Etienne Thiry, Professor, DVM, MS, PhD, DECVPH, is a professor at the University of Liège, Belgium, and has held several positions in the university's laboratory of veterinary virology. He graduated from the University of Liège as a doctor of veterinary medicine in 1980, after which he obtained a master's degree in molecular biology at the University of Brussels and a doctorate in veterinary sciences, also at the University of Liège. In 1992, he graduated in medicine and epidemiology at the University Pierre and Marie Curie in Paris and subsequently became a diplomate of the European College for Veterinary Public Health. Prof. Thiry is a member of the National Committee for Microbiology of the Belgian Royal Academy and is the president of the Belgian Association of Animal Health and Epidemiology. He previously served on the board of the European Society for Veterinary Virology.

Heinz Mehlhorn, Professor, PhD, is Director of the Institute for Zoomorphology, Cytology, and Parasitology at the University of Düsseldorf, a position to which he was appointed in 1995. He completed his studies in biology and received his PhD at the University of Bonn (Germany) in 1971, then served as a professor at the Universities of Düsseldorf and Bochum. Prof. Mehlhorn is presently the European representative of the World Federation of Parasitologists and has been honored with national and international medals of acknowledgment. He has 25 patents, and his work has been published in more than 20 books and more than 200 scientific publications. Prof. Mehlhorn is also founder and scientific director of the company Alpha-Biocare.

Birgit Makoschey, DVM, PhD, is certified as a veterinary microbiologist (Fachtierarzt für Mikrobiologie) and holds the position of International Technical Manager for Ruminant Biologicals at the International Marketing Department of Intervet/Schering-Plough Animal Health. She graduated from the veterinary school in Hannover, Germany. After completing her thesis in the Department of Virology at the German Primate Center, she had a 2-year assignment at the Laboratoire de Génétique des Virus, Centre National de la Recherche Scientifique, France. In 1996 Dr. Makoschey accepted the position as project leader in the Virological R&D Department of Intervet International, Boxmeer, The Netherlands. For nearly 10 years she has worked with various bovine viruses. Dr. Makoschey is the author of numerous scientific publications.

Bluetongue: The Virus and the Infection in Cattle and Small Ruminants

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Abstract

Bluetongue is a noncontagious vectorial disease of ruminant species and is seen worldwide. The vector is an insect of the family *Ceratopogonidae* and the genus *Culicoides*. After infection by an insect bite, the virus replicates in the regional lymph nodes and induces a viremia. The disease is seasonal, mainly in hot, humid areas near stagnant water. Sheep are the main host, but the infection also occurs and leads to subclinical infection in cattle, free-living ruminants, and goats. Signs of the infection are usually subclinical in cattle, which are transient reservoirs for the virus, although serotype 8 that recently emerged in Europe exhibits a more important virulence. The vascular endothelium is less affected in cattle; but, in sheep, lesions in the endothelial cells of small blood vessels provoke edema, hemorrhages, and, in severe cases, vascular thrombosis and ischemic necrosis of the affected tissue. Cell-associated viremia of long duration is characteristic of the disease. Bluetongue virus infection is not lifelong persistent, however. Besides transmission by the insect vector, the virus can be transmitted vertically in utero. Cases of abortion and fetal malformations due to bluetongue infection occur sporadically.

Introduction

Bluetongue is a noncontagious vectorial disease that is distributed worldwide and affects ruminant species.[1] The virus belongs to the genus *Orbivirus* of the family *Reoviridae*. The vector is an insect of the family *Ceratopogonidae* and the genus *Culicoides*.[2,3] Found in various parts of the world, the infection is usually inapparent in cattle, which act as transient reservoirs for the virus. Serotype 8, however, which recently caused infection in northern Europe, exhibits a more important virulence in cattle.[4] Sheep are the main host of the virus, but the infection also occurs and leads to subclinical infection in cattle, free-living ruminants, and goats. Again, clinical signs have been observed in cattle and goats during the recent serotype 8 outbreak in northern Europe.

Usually, in countries where the infection is endemic or frequently occurs, local ovine breeds are more resistant to the viral infection than imported or non-native breeds. Cervidae are also infected by a closely related orbivirus responsible for epizootic hemorrhagic disease. Some deer species exhibit a high susceptibility to bluetongue virus, eg, the white-tailed deer (*Odocoileus virginianus*). Many other ruminants can also be found infected with bluetongue virus. For example, yaks infected with serotype 8 bluetongue virus during the 2006 outbreak in northern Europe were severely affected.[5]

Bluetongue infection is usually subclinical and inapparent in cattle, although some serotypes, such as serotype 8, show more important virulence.

Structure and Replication of the Virus

The virus contains 2 capsids enclosing a core consisting of 10 segments of double-stranded RNA that encode the 7 structural proteins (VP1 to VP7) and 4 nonstructural proteins (NS1 to NS3, NS3A). ***See Figure 1.*** The external capsid contains the VP5 and VP2 proteins that are responsible for serotype specificity. Neutralizing antibodies are mainly directed to these 2 proteins. The internal capsid consists of the VP7 protein, which is the specific group antigen. VP1 protein is present in the core of the virus, and its function is RNA polymerase. The genome is segmented, and reassortment of segments can occur during co-infections. In addition, the viral genome has a high rate of mutations contributing to an antigenic drift. The genetic variability of bluetongue virus, such that 24 serotypes exist, is due to these characteristics. Among these 24 serotypes the serotypes 1, 2, 4, 9, and 16 were reported between 1998 and 2006 in Mediterranean Europe. Serotype 8 was responsible for the epidemic in northern Europe in 2006.[3,4]

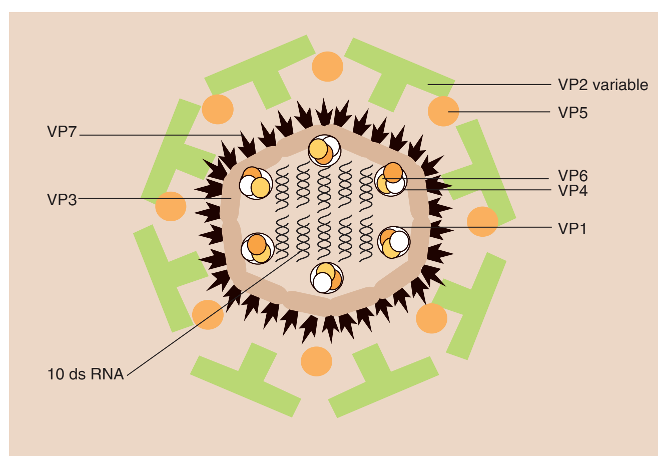

Figure 1. Schematic representation of the bluetongue viral structure.
VP = viral protein, ds RNA = double-stranded ribonucleic acid.
Reproduced from the article: Thiry E, Gauthier J-F. La fièvre catarrhale ovine est-elle installée durablement? *Le Point Vétérinaire.* 2008;281:57-61.

The virus persists in the culicoid midges during their life span. After a blood meal, the virus passes through the intestinal wall and is distributed via the hemocoel into the body of the insect to reach various tissues and then the salivary glands where it continues to replicate. The virus is subsequently excreted in the insect's saliva. Viral transmission therefore occurs almost solely by insect bite. The vector obtains the maximal infective capacity 10 days after having absorbed blood from the viremic animal.

After infection by an insect bite, the virus replicates in the regional lymph nodes. It disseminates to infect the vascular endothelium and macrophages as well as dendritic cells in various organs. In the blood, the virus is adsorbed at the surface of erythrocytes and platelets, whereas it replicates in monocytes and lymphoblasts. The infectious virus is enclosed in erythrocytic and lymphocytic plasmic membrane invaginations; and, therefore, viremia is persistent in the presence of neutralizing antibodies.

Viral transmission occurs almost solely by insect bite, with the vector obtaining maximal infective capacity 10 days after absorbing blood from a viremic animal.

Clinical Expression of the Disease

There is a great difference in expression of the disease in cattle and sheep: in contrast to sheep, the vascular endothelium in cattle is much less infected. In sheep, lesions in the endothelial cells of small blood vessels provoke vascular thrombosis and ischemic necrosis of the affected tissue. These lesions result in buccal ulcers, inflammation of the coronary band, muscular necrosis, and extravasation leading to facial and pulmonary edema and pleural and pericardiac effusions.[6,7]

Expression of bluetongue disease differs greatly between cattle, which are much less infected by the virus at the vascular endothelium, and sheep, which develop lesions in the endothelial cells of small blood vessels resulting in tissue thrombosis and necrosis.

During the serotype 8 outbreak that occurred during and after August 2006 in northern Europe, clinical signs were observed in cattle ***(Figures 2 and 3)*** and sheep as well as in goats.[8] The confusion of the clinical signs of bluetongue with other major diseases of cattle, like malignant catarrhal fever and mucosal disease, needs the very careful attention of the veterinary practitioner.[9] ***See Figures 4 and 5***.

Viremia of Long Duration

Cell-associated viremia of long duration is characteristic of bluetongue disease. The cell-free viremia is transient. The high level of viral infection and long-lasting viremia increase the risk of infection from culicoid vectors. Neutralizing antibodies appear after 14 days but do not eliminate the virus, which is protected by its association with blood cells. At the beginning of the viremia, the virus is associated with vari-

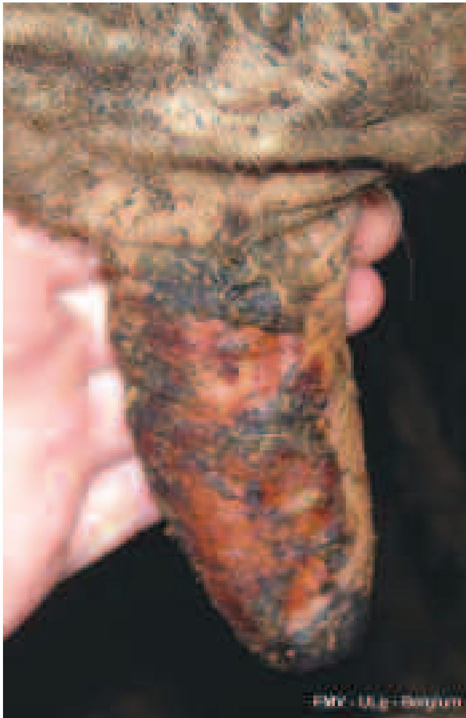

Figure 2. Teat with petechiae and crusts.

Figure 3. Facial edema resulting from extravasation.

Figure 4. Ulcers in the nasal mucosa.

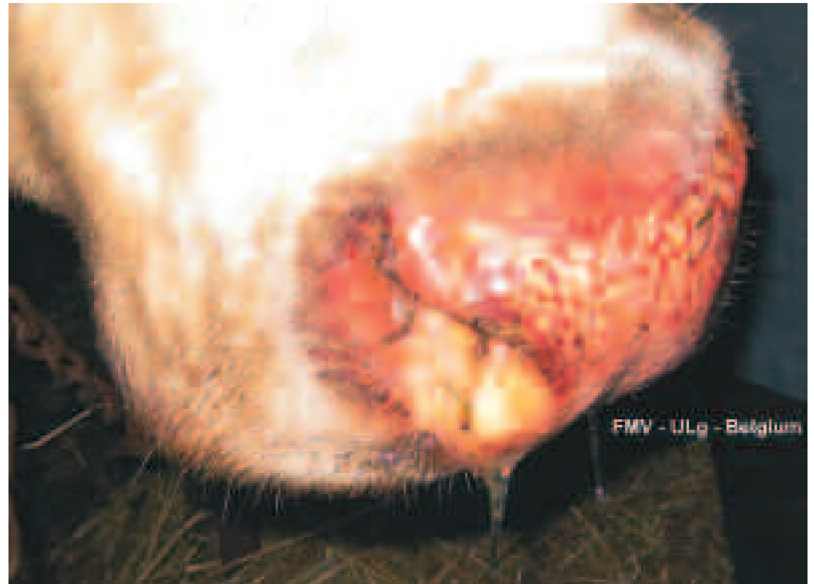

Figure 5. Ulcerations and crusts in the nasal plane, with nasal discharge.

ous blood cells. Subsequently, the viremia is nearly exclusively associated with blood erythrocytes. These cells do not, however, contain the necessary machinery for viral replication.[6]

Bluetongue virus infection is not persistent lifelong. The duration of the viremia is in part associated with the life span of erythrocytes; and, therefore, the viremia is of longer duration in cattle than in sheep. The pathogenesis varies depending on the virus serotype. In experimental conditions, the viremia lasts for 14 to 45 days in sheep and up to 31 days in goats. Reverse transcriptase polymerase chain reaction is used to detect the viral genome. With this technique the duration of the viremia is much longer. The duration of the viremia that is capable of infecting hematophagous vectors is about 60 days, although probably much shorter in

field conditions. The viremia can last for more than 100 days in cattle, which is, therefore, longer than in sheep. Infected bulls may excrete the virus in sperm and become carriers for long periods.[7]

Duration of viremia is associated with the life span of erythrocytes and is, therefore, of longer duration in cattle than in sheep.

Disease Transmission Factors

In addition to transmission by the insect vector, the virus can be transmitted vertically in utero. Cases of abortion and fetal malformations due to bluetongue infection occur sporadically in ruminants. In serotype 8 epidemics in northern Europe, however, the incidence of abortions, congenital abnormalities, and reproduction failures is increased in cattle and sheep. This feature is currently being carefully investigated by several laboratories.

Transplacental passage of virus produces variable clinical signs depending on the period of gestation when infection occurs. During the first trimester of gestation, embryonic and fetal deaths occur. Infection during the second third of pregnancy can provoke congenital abnormalities, such as hydranencephaly and retinal dysplasia, which are due to destruction of neuron and glial cell precursors by the virus before these cells migrate to different areas of the brain. During the last trimester of gestation, the fetus develops an immune response and eliminates the infection. Abortion is rare compared to congenital abnormalities. Some abortions are nonspecific and are a direct stress-related consequence of infection in the ewe.[10] During the serotype 8 epidemics in northern Europe, loss of fertility was noticed in both sheep and cattle. Especially, bluetongue infection was associated with poor quality of sperm.

Vectors are midges of the genus *Culicoides*, including *C. imicola* in Africa and Mediterranean Europe, *C. sonorensis* in North America, *C. insignis* and *C. pusillus* in South America, and *C. brevitarsis* in Australia. In Europe, *C. obsoletus* and *C. scoticus* have been identified in central Italy and *C. pulicaris* in Sicily. *C. dewulfi* and other culicoids of the complex *obsoletus* are recognized as vectors in northern Europe.[11] ***See table***.

Bluetongue infection occurs after introduction of vectors or infected sheep in a virus-free area where the vector is indigenous. Subclinical infection commonly occurs in cattle

Bluetongue Disease Vectors by Global Region

Region	***Vector***
Africa Mediterranean Europe	*Culicoides imicola*
Australia	*Culicoides brevitarsis*
Europe—Italy (central)	*Culicoides obsoletus* *Culicoides scoticus*
Europe—Northern	*Culicoides dewulfi* *Culicoides obsoletus*
Europe—Sicily	*Culicoides pulicaris*
North America	*Culicoides sonorensis*
South America	*Culicoides insignis* *Culicoides pusillus*

and goats, and these species could serve as reservoirs for the infection. After the disease is endemic, clinical signs are seen mainly in susceptible imported sheep. The distribution of virus depends on the presence of culicoid vectors. Therefore the disease is seasonal and seen mainly in hot, humid areas near stagnant water ponds. The disease occurs mainly at the end of summer or beginning of winter in temperate regions and throughout the year or, more often, in spring and at the beginning of summer in subtropical areas.[11]

In the absence of transovarial transmission of the virus in insects, other mechanisms are suggested to explain the phenomenon of overwintering, that is, viral survival in the winter period and even during prolonged periods over 9 to 12 months when there is absence of vector adults. Overwintering is dependent on the establishment of chronic infections in sheep and cattle. The $\gamma\delta$ T lymphocytes are associated with persistent infection in sheep.[12]

Subclinical bluetongue infection is common in cattle and goats, which could serve as reservoirs. After the disease is endemic, clinical signs are seen mainly in susceptible imported sheep.

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Midges As Vectors of Viruses of Bluetongue Disease and Measurements of Protection

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Abstract

Bluetongue disease and the family Ceratopogonidae (especially the *Culicoides* genus) were not considered of particular interest in Europe until the central European outbreak in 2006. *Culicoides imicola*, the main vector in Africa and southern Europe had not been detected in central Europe. With the outbreak of bluetongue disease in The Netherlands, Belgium, and Germany in 2006, however, it became clear that this virus-derived disease has an enormous impact on the livestock in all of Europe. The tiny species, *Culicoides obsoletus*, was found to be most common and proved to be the vector. Control of the vector through treatment of the animals with insecticides is one method of disease control, as each avoided bite decreases the risk of virus transmission. Besides insecticidal treatment, protection of ruminants against bluetongue virus transmission should include vaccination.

Introduction

Until 2006 neither bluetongue disease nor the blood-sucking family Ceratopogonidae (especially the members of the genus *Culicoides*) had been considered in Europe with peculiar interest. Bluetongue was a disease of ruminants in Africa, and only a few outbreaks with other serotypes occurred at the northern border of the Mediterranean Sea. Furthermore, the belief was that there was no danger for central and northern Europe, as the main vector in Africa and southern Europe—*Culicoides imicola*—had not been seen in central Europe. As soon as the outbreak of bluetongue disease occurred in The Netherlands, Belgium, and Germany in 2006, however, it became clear that this virus-derived disease has an enormous impact on the livestock in all of Europe. The disease spreads quickly with high morbidity leading to considerable death rates in cattle (3% to 4%) and in sheep (up to 40%).[1-3]

The 2006 outbreak in The Netherlands, Belgium, and Germany made it clear that the high morbidity and considerable death rates associated with bluetongue disease have an enormous impact on the livestock in all of Europe.

Monitoring of the years 2006 and 2007 with respect to vectors and their infectivity demonstrated that the very tiny (0.8 mm) species, *Culicoides obsoletus* ***(Figure 1)***, was the most common (about 75% to 95% of the specimen catch-

Figure 1. Light micrograph of a female *Culicoides obsoletus* midge.

The tiny *Culicoides obsoletus* midge proved to be the bluetongue disease vector, but specimens of the much larger *Culicoides pulicaris* midge were also detected by polymerase chain reaction on bluetongue virus serotype 8.

es) and proved to be the vector, as the Friedrich Löffler Institute on the Island of Riems detected the virus also in non-fed females.[2] In insect catches of the year 2007 this was confirmed in probes all over Germany; however, several specimens of the much larger species, *Culicoides pulicaris* (up to 4 mm long), were detected to be polymerase chain reaction (PCR)-positive on the bluetongue virus of the serotype 8, for which the predominant hosts are antelopes and other local ruminants of South Africa. Because to that point only fed females of *C. pulicaris* were found PCR-positive, the finding might have been due to the virus load of ingested virus-positive cattle blood. Thus, the vectorship of *C. pulicaris* is not yet completely proven.

Vector Proliferation and Spread of Disease

Culicoides obsoletus was shown to be active in Germany almost year-round. Apparently, the species breeds close to the animals, possibly also inside their stables. Furthermore, the life span may reach up to 4 months (compared to 1.5 months in other species). Females of *C. obsoletus* therefore have good opportunities as vectors—especially from August until October when they occur in huge numbers (during monitoring, up to 50,000 specimens were caught in traps within a few days).

The spreading of bluetongue disease started in 2006 in the western region of Germany, and within 4 weeks the virus crossed the Rhine eastward and in 2007 reached the eastern border of Germany. This process could be explained by propagation via wind-driven, infected *Culicoides* specimens, as several authors reported that the tiny midges may be displaced within 1 night for 6 to 8 kilometers, even if a rather light wind blows. The spreading of the virus southward to Bavaria, Baden-Württemberg, Luxembourg, Saarland, and large regions of northern and central France is difficult to explain, however. Probably spreading involves additional factors that support the epidemical spreading of the disease. Animal transportations over shorter and longer distances are surely important, but additional factors that allow a concentric spreading around a farm with infected animals must also be involved. Thus, mechanical infections via contaminated mouthparts of insects other than midges seem possible, as large numbers of licking flies or biting horseflies were attracted by the open wounds of heavily infected animals.

Control of the *Culicoides* spp. vector by treating animals with insecticides is a likely method of controlling bluetongue disease, as each insect bite avoided decreases the risk of virus transmission.

Insecticidal Control of the Vector

Control of the vector through treatment of the animals with insecticides is a distinct possibility to control bluetongue disease, as each avoided bite decreases the risk of virus transmission. Furthermore, the pyrethroid insecticides kill the attacking females soon after their contact with treated hair (knockout effect). Thus, those female midges are excluded from egg production, and the potential reproduction rate of possible vectors is kept low in the surroundings of treated animals. Studies have been performed to determine the efficacy of deltamethrin (Butox®—Intervet/Schering-Plough Animal Health, The Netherlands) against *Culicoides* spp.

Although the deltamethrin product was poured onto the back of the animals ***(Figures 2 through 5)***, experiments in cattle and sheep showed that the insecticide creeps also to the hair of the legs and belly, which are predominant biting sites for the *Culicoides* sp. females. For sheep, however, it is necessary to deliver the insecticide directly onto the skin by spreading the thick fleece before application.

At intervals of 7 days (ie, on days 7, 14, 21, 28, 35) hair was trimmed off the legs of treated cattle and sheep and brought into contact with living midges, which had been caught the night before in ultraviolet light-exposing traps, because the midges show their main activity from 4:00 pm to 12:00 am. The contacts between the midges and the hair of treated animals were regulated to last for 15 seconds, 30 seconds, and 1 or 2 minutes. In controls using untreated hair the midges showed no change for more than 3 days.

Text continues on page 15

Figure 2. Cutting of hair from the leg of a sheep.

Figure 3. Spreading of the fleece of a sheep and treatment with Butox®.

Figure 4. Removal of hair from the leg in cattle.

Figure 5. Application of Butox® on the back of cattle.

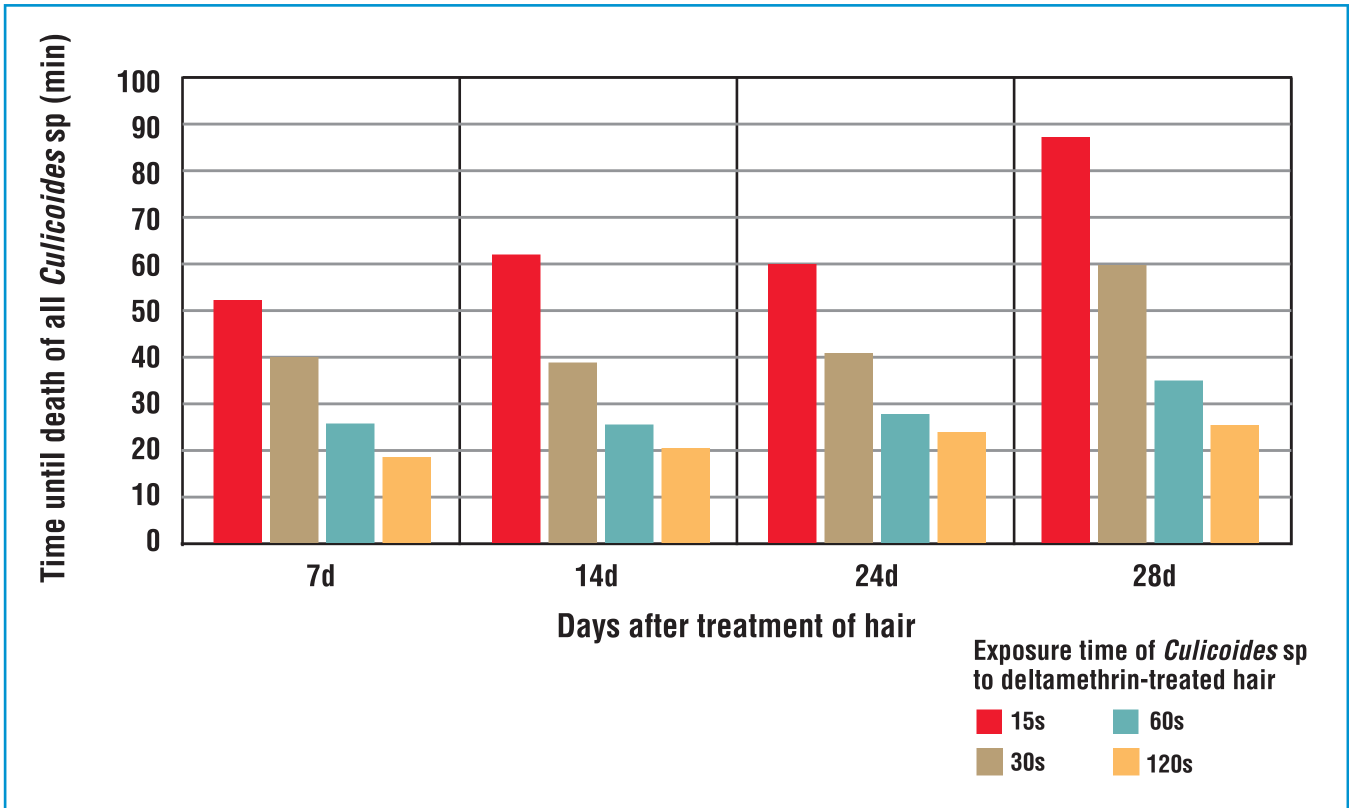

Figure 6. Killing effects of deltamethrin-treated hair from sheep on specimens of *Culicoides* sp.

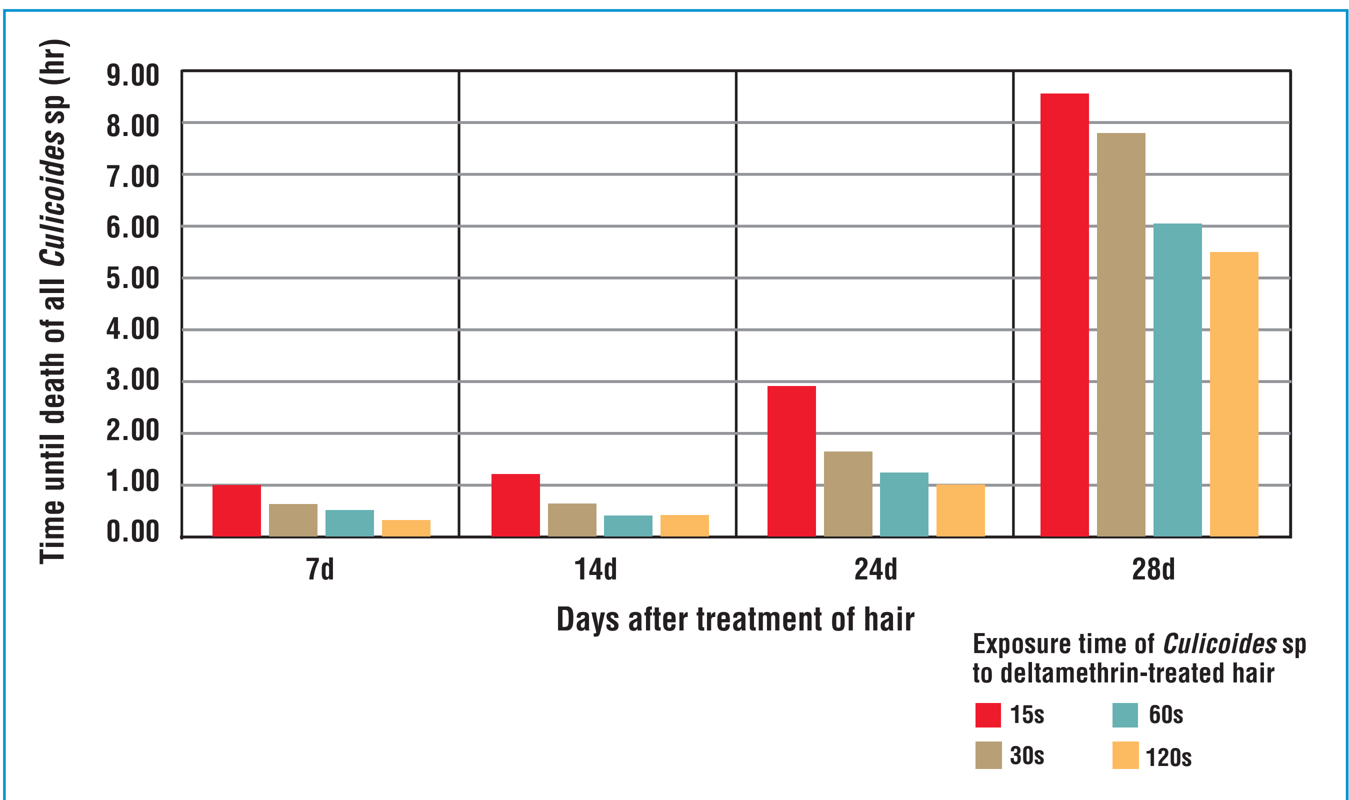

Figure 7. Killing effects of deltamethrin-treated hair from cattle on *Culicoides* sp.

The results showed that the insecticide killed the specimens of *Culicoides* of both species (*C. obsoletus* and *C. pulicaris*) even 4 weeks after treatment of the animals, if contact to treated hair was as limited as only 15 seconds ***(Figures 6 and 7)***. Because contact with treated hair introduced immediate paralysis in the insects, they likely lost the ability to bite and, thus, were unable to act as transmitting agents for disease.

Considerations for Ruminant Protection

Because the ruminant's body may have sites that receive low amounts of insecticide (eg, along the udder, nose, ears), a field study was started in April 2008 to compare the seroconversion rates of a treated group of sheep versus an untreated group. Regardless of the study outcome, the protection of ruminants against bluetongue virus transmission should include vaccination as well as insecticides.

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Experimental Evaluation of an Inactivated Vaccine Against Bluetongue Virus Serotype 8

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Abstract

Following the first outbreaks of bluetongue virus (BTV) serotype 8 in Europe in 2006, a project was initiated to develop an inactivated vaccine against the virus. A number of studies have been performed with experimental vaccine preparations to establish the safety and efficacy in cattle and sheep. The vaccine was found to be well tolerated by young calves and lambs even after overdose vaccination followed by two repeated injections at 2-week intervals. Based on vaccination-challenge studies in lambs, the antigen content was set at 500 antigen units (AU)/mL for the BTV 8 vaccine. In sheep, a basic vaccination course consisting of a single subcutaneous injection of 1 mL was found to confer complete protection against viremia. For cattle, a two-dose primary vaccination course is recommended.

Introduction

The 2006 outbreaks of bluetongue virus (BTV) serotype 8 were fairly unexpected, although some concerns had been expressed beforehand that serotypes of BTV other than those already determined might expand toward western-central Europe. Therefore, neither the vaccine strain nor the challenge model for BTV 8 nor methods for laboratory testing were available at the authors' laboratory. Given the urgent need for a vaccine, the disadvantages of modified live vaccines, and the regulatory difficulties to license genetically modified vaccines at that time, only an inactivated BTV 8 vaccine was a feasible option for use in Europe.

Three studies were performed to establish the minimum effective antigen dose in sheep, the safety of the BTV 8 vaccine in calves and sheep, and vaccine efficacy in calves.

The studies described herein were performed to (a) determine the minimal effective antigen dose in sheep, (b) to establish the safety of the BTV 8 vaccine after overdose and repeated dose in calves and sheep, and (c) to determine the vaccine's efficacy in calves. ***See Table 1***.

Materials and Methods

Vaccine

The BTV 8 vaccine strain was isolated at the Intervet research and development facilities in Boxmeer. That strain originated in an infected cow from The Netherlands. The amount of antigen per dose differed among three studies:

- ***Graded dose study (sheep):*** in the graded dose study in sheep, vaccine preparations with 1,000, 500, and 250 AU/mL were used.
- ***Safety study (calves and sheep):*** the vaccine used in the safety study contained 3,000 AU/mL.
- ***Efficacy study (calves):*** the vaccine batch used in the efficacy study in calves was blended at a concentration of 500 AU/mL.

Table 1. Experimental Studies of BTV 8 Vaccine Safety and Efficacy in Cattle and Sheep

Study Type	*Species / No. of Animals*	*Dosing*	*Route*	*BTV 8 Challenge*	*Outcomes*
Graded dose	Sheep / 56	8 treatment groups: 1, 2, and 3 vaccinated twice at 3-week interval with 1,000, 500, 250 AU, respectively; concurrent with second vaccination 4, 5, and 6 single vaccinated with 1,000, 500, 250 AU, respectively; 7 and 8 were saline controls	SQ	Live BTV 8 3 weeks after second vaccination	Humoral response in most animals after first dose and in all animals after second dose; BTV 8-specific antibodies in controls after challenge; all controls viremic from 2-4 days after challenge; with 2 exceptions, no viremia seen in any other vaccinated animals
Safety	Cattle / 11 Sheep / 20	Overdoses: initial calves 4 mL, lambs 2 mL then calves 2 mL lambs 1 mL at 2 and 4 weeks	SQ	—	All vaccinated animals showed seroconversion against BTV 8; all unvaccinated animals remained seronegative; significant differences in body temperature between vaccinated and unvaccinated animals after first and second but not third treatments; some granulomatous inflammation at infection sites
Efficacy	Cattle / 13	1 mL BTV 8 vaccination twice at 3-week interval; 2 mL saline for controls	SQ	Live BTV 8 3 weeks after second vaccination	All calves seronegative for BTV 8 after first vaccination; BTV 8-specific antibodies in vaccinated animals after second vaccination and increased titers after challenge; viral RNA found in all controls 6 days after challenge and on; virus load and duration clearly reduced in vaccinated animals

The same adjuvant, a combination of saponin and aluminum hydroxide ($Al(OH)_3$), was used at the identical concentration in all vaccine batches tested.

Experimental design

Efficacy of different antigen concentrations in sheep

This study was performed at the CReSA (Centre de Recerca en Sanitat Animal, Barcelona) on 56 conventional sheep that were 5 weeks of age ***(Table 2)***. At the start of the study, all lambs were free of antibodies against BTV. They were randomly allocated to 1 of the 8 treatment groups of 7 animals each. The lambs in groups 1, 2, and 3 were vaccinated twice at a 3-week interval with a vaccine preparation containing 1,000, 500, and 250 AU, respectively. At the time of the second vaccination groups 4, 5, and 6 were single vaccinated with the same vaccine preparations containing 1,000 AU (group 4), 500 AU (group 5), and 250 AU (group 6). Groups 7 and 8 served as controls. The control groups were vaccinated twice (group 7) or once (group 8) with saline. All vaccinations were performed subcutaneously. Three weeks after the (second) vaccinations, all 8 groups were challenged subcutaneously with live BTV 8.

To study effects of antigen concentrations, 56 lambs were randomly allocated to 1 of 8 treatment groups and vaccinated subcutaneously either once or twice at a 3-week interval with vaccine preparations containing different AU or saline (controls).

Safety studies in calves and lambs

Following the requirements for licensing of inactivated vaccines for cattle and sheep, the safety of the BTV 8 vaccine

Table 2. Number of Lambs Tested Positive for Viremia (×/7) After Vaccination and Challenge Virus Infection at Different Times Postinfection*

Group	D0	D2	D4	D6	D8	D10	D12	D14	D16	D18	D20	D22	D24
2×1,000	0	0	0	0	0	0	0	0	0	0	0	0	0
2×500	0	0	0	1	0	0	0	0	0	0	0	0	0
2×250	0	0	0	0	0	0	0	0	0	0	0	0	0
1×1,000	0	1	1	1	1	1	1	1	0	1	1	1	1
1×500	0	0	0	0	0	0	0	0	0	0	0	0	
1×250	0	0	0	0	0	0	0	0	0	0	0	0	
2xsaline	0	6	7	7	7	7	7	7	7	7	7	7	7
1xsaline	0	5	7	7	7	7	7	7	7	7	7	7	7

**Vaccine preparations contained 1,000, 500, or 250 antigen units (AU) as indicated.*

after overdose and repeated doses was established in both species. All vaccinations were given subcutaneously. Eleven conventional calves at the age of 4 to 5 weeks and 20 lambs at the age of 5 to 10 weeks were included in the study. The animals were vaccinated first with an overdose (4 mL for calves or 2 mL for lambs) of the vaccine (n=6 calves and 11 lambs) or saline (n=5 calves and 9 lambs). After 2 and 4 weeks, vaccination was repeated with a 2 mL dose in calves and a 1 mL dose in lambs.

Efficacy study in calves

A vaccination-challenge study was performed to determine the protection of calves against BTV 8 challenge. Thirteen conventional calves at the age of 1.5 to 3.5 months were included in the study. At the start of the study, all calves were free of antibodies against BTV. The calves were allocated at random to one of two treatment groups. The calves in group 1 (n=7) were vaccinated twice at a 3-week interval with a 1 mL dose of the BTV 8 vaccine. A second group of 6 calves served as controls and were vaccinated with 2 mL of saline. All vaccinations were performed subcutaneously. Three weeks after the second vaccination, all calves were challenged subcutaneously with live BTV 8.

A study of vaccine efficacy was performed on two calf treatment groups—vaccinated versus saline controls—to determine protection against BTV challenge.

Parameters

Body temperatures were recorded daily after each vaccination and the challenge infections. The animals were observed daily for general health and after challenge more specifically for BTV 8-specific signs. In the safety studies, local injection-site reactions were monitored after each injection. Two weeks after the third vaccination, animals were necropsied and the injection sites were examined macroscopically and microscopically.

Blood samples were collected at preset times to determine the BTV 8 antibody response after vaccination and challenge. In the two efficacy studies, additional blood samples were taken just prior to challenge and then every 2 or 3 days during 3 weeks to determine BTV 8 viremia by real-time polymerase chain reaction.

Results

Results of the graded dose study in lambs

All lambs were negative for BTV antibodies at the beginning of the study. A BTV-specific humoral response was detected in most animals after a single dose and in all animals after the second dose of the vaccine. A clear anamnestic response was detected after challenge of the vaccinated sheep. Moreover, all control animals developed BTV-specific antibodies after challenge.

After vaccination, especially after the second dose, some lambs displayed a temporary increase in body temperature during 1 or 2 days. The unvaccinated animals exhibited an increase in body temperature with peak temperature reached between 5 to 6 days after challenge and lasting 3 to 4 days. Both single and double vaccination with any of the different doses of antigen prevented this increase.

The most important criterion for efficacy was the protection against BTV challenge virus viremia. All control lambs were viremic from 2 or 4 days after challenge onward. With the exception of one lamb vaccinated twice with the 500 AU preparation during 1 day and another lamb vaccinated once with 1,000 AU, no viremia was detected in any of the remaining animals.

Protection against BTV challenge virus viremia is the most important criterion for efficacy.

Results of the safety study in calves and lambs

All vaccinated animals showed seroconversion against BTV 8 at the end of the experiment, and the unvaccinated animals remained seronegative. The vaccinated animals displayed a temporary increase in body temperature after the vaccinations. In calves, the temperature differences between vaccinated and control animals were significant after the overdose vaccination (Δ 0.7 °C) and the second vaccination (Δ 1.2 °C) but not after the third vaccination (Δ 0.5 °C). Also in the lambs, temperature differences between the vaccinated and control groups were significant after the overdose vaccination (Δ 0.5 °C) and the second vaccination (Δ 0.7 °C) but not after the third vaccination (Δ 0.2 °C). No other systemic reactions were observed after repeated vaccination with an overdose and repeated doses of the inactivated BTV 8 vaccine.

Both the calves and the lambs developed moderate swellings at the injection sites after the overdose and the repeated vaccinations. The swellings were initially soft but decreased in size and changed into hard swellings in time. Most important, they were at no time painful. At necropsy, the local reactions were still visible. The tissue alterations were consistent with a granulomatous inflammation.

Results of the efficacy study in calves

All calves were found to be seronegative for BTV 8 at the time of the first vaccination. The vaccinated animals developed BTV 8-specific antibodies after the second vaccination, and titers increased further after challenge. In the unvaccinated animals, a detectable BTV 8 antibody response was measured from 2 weeks after challenge onward.

Only the unvaccinated animals developed an increase in body temperature about 1 week after infection, which can be regarded as related to the challenge infection. After challenge virus infection, viral RNA was detected in blood samples from all control animals from 6 days after challenge onward. Virus load and duration of viremia were clearly reduced in the vaccinated animals.

Conclusions

Based on the results obtained after overdose vaccination of 4-week-old to 5-week-old calves and 5-week-old to 10-week-old lambs followed by two repeated injections at a 2-week interval, it was concluded that the BTV 8 vaccine was well tolerated by the animals.

In the vaccination challenge study in lambs, all three antigen doses tested (1,000, 500, and 250 AU) were found to be equally effective. Subsequently, the vaccine dose was

Results of overdose and repeated vaccination indicated that BTV 8 vaccine was well tolerated by all animals tested.

set at 500 AU/mL. Moreover, one vaccination was found to be as effective as two and, therefore, a single-dose vaccination course can be recommended for sheep. For cattle, a two-dose vaccination regimen is recommended.

In summary, the studies performed to date with the BTV 8 vaccine have demonstrated that the vaccine is safe for use in cattle and sheep even at a young age. Moreover, the vaccine was found to be highly efficacious in reducing challenge-virus viremia, which is the key factor in controlling the BTV 8 epidemiology.

Outlook

To be able to apply for a full license according to the European Standards, additional studies have been initiated or are in preparation. Those studies will address the following questions:

- ▲ Duration of immunity and immunity in face of maternal antibodies.
- ▲ Safety for pregnant cows and ewes.
- ▲ Safety and efficacy of the vaccine under field conditions.
- ▲ Stability of the product.

Notes

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