ANALYSIS OF MYXOMATOSIS OUTBREAKS ON SPANISH RABBIT FARMS

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ABSTRACT

Myxomatosis is a fatal disease caused by myxoma virus (MV), a member of the poxviridae. Although effective vaccines are available, outbreaks on Spanish rabbit farms cause large economic losses. In order to study the causes of these outbreaks we have focused our attention on characterizing circulating viruses and analyzing the immune response of vaccinated rabbits in both rabbitry and laboratory conditions. Using a network of veterinary surgeons and technicians we received samples suspected of containing myxoma virus from 22 affected rabbitries. Firstly we used PCR to confirm the presence of myxoma virus, we next targeted 3 genomic regions by PCR to obtain sequence data in order to typify the virus present. In a selected number of samples the virus was isolated by cultivation in tissue culture. Using the sequence data we selected a virus isolate as representative of the current circulating field viruses causing outbreaks on Spanish rabbit farms. The viral strain Granada -05/09 was characterized for its virulence in New Zealand white rabbits and shown to be of virulence grade A (maximum virulence grade).Next, we measured seroconversion in vaccinated rabbits under both farm and laboratory conditions. Using anti-MV antibody levels as an indicator of immune response we compared methods of vaccine administration (intradermic or subcutaneous). In laboratory rabbits we tested the efficacy of a current vaccine strain against the MV Granada -05/09 strain. Our findings indicate that MV vaccines are capable of protecting against currently circulating strains but the levels of seroconversion after vaccination vary greatly and depend on the method (route) of vaccine administration.

Key words: Myxoma virus, characterization, sequence, antibody response.

INTRODUCTION

Myxoma virus (MV) is the cause of myxomatosis. Classic myxomatosis is spread by insect vectors and contact with infected individuals. Prevention relies on good hygienic practises, control insect vectors and vaccination. There are currently two types of MV vaccine. Homologous live vaccine and heterologous live (Shope fibroma virus) vaccine. Despite these effective vaccines the disease is a recurrent problem on rabbit farms throughout Spain and Europe (Farsang et al., 2003; Kritas et al., 2008; Barcena et al., 2000). The problem calls into question the efficacy of current MV vaccines. One possible cause maybe due to the emergence of new strains of MV which evade protective immune responses generated by vaccination. Another possible cause is the improper execution of vaccine strategies.

Traditionally myxoma viruses circulating in wild rabbit populations have been characterised by their virulence in experimental infections (Barcena et al., 2000). MV strains are classified into 5 virulence grades (A to E, A being most virulent and E the most attenuated) based on the mean survival time of rabbits after infection (Fenner and Marshall 1957). The genetic study and characterisation of myxoma virus genomes is difficult due to their large size (161.8 kb) (Cameron et al., 1999). Previous studies

relied on RFLP analysis (Saint et al., 2001;Kerr et al., 2003;Labudovic et al., 2004), while recent studies of field strains of MV have focussed on sequencing individual genes or gene fragments (Alda *et al.*, 2009; Muller *et al.*, 2010). However, to date there is no way to define virulence grades based on sequence analysis. We have partially characterised the genomes of 3 vaccines strains (unpublished data; Cavadini *et al.*, 2010 and Guerin *et al.*, 1998) to better understand MV virulence factors and to be able to differentiate between genomes of vaccine and wild-type strains.

Although vaccine protocols exist there is no effective global vaccination strategy used on Spanish rabbit farms. The use of either homologous or heterologous vaccines, the way vaccines are administered and how often, vary from farm to farm. Often the vaccine is used in response to on-going outbreaks, which due to the immune suppression caused (particularly in young rabbits) may actually do more harm than good. A study comparing and combining vaccination with heterologous and homologous vaccines showed that the strategy employed effects protection (Marlier *et al.*, 2000).

Much is known about the immune response to MV and the way MV manipulates this response (for review see Zuniga 2002) and it is considered that the cellular mediated immune response is responsible for virus clearance. However, currently the only commercially available method of detecting exposure to MV and/or successful vaccination is by the detection of antibodies in sera and to date the challenge of vaccinated rabbits is the only reliable method for measuring whether or not a vaccine is effective against particular strains of MV.

Objectives: to characterise MV field isolates from farm outbreaks; to differentiate them from classic and vaccine strains, to compare methods (route) of vaccine administration and to test the efficacy of current vaccine strains against challenge with current isolates.

MATERIALS AND METHODS

Virus and cells

Myxoma virus Lausanne strain (Lu), vaccine strains and wild type isolates were grown and titered in RK13 cells as described in Dalton et al., 2010.

The MV strain used in the virulence grading study was isolated from eyelid samples received from a rabbit farm in 2009. The isolate is identified by its region of origin and the month and year of isolation – Granada-05/09. Three vaccine strains were bought from the three main myxoma virus vaccine producing companies in Spain and cultivated in the cell line RK13 using the same culture conditions as described for the wild type strain.

DNA extraction and PCR DNA extractions were performed as described by Dalton et al., (2010). The oligonucleotides used for the PCR amplification and sequencing were designed based on the sequence of Lu genomic sequence and PCRs carried out as described by Dalton et al. (2010).

Experimental vaccination and infection Rabbits used in the experimental vaccinations and infections were New Zealand White. Animal experiments were carried out at the University of Leon in the Animalario facility. The experiment was approved by the ethical committee board of the University of Leon. A total of 18 rabbits were used. Six were infected experimentally, while 12 were vaccinated and one month later used in challenge experiments.

ELISA Seroconversion was checked using a commercial ELISA kit - CIVTEST (Hipra) following the manufacturer´s instructions.

Sequence analysis

The sequencing service at the Universidad de Oviedo was used to sequence all gel purified PCR products and Chromas LITE freeware (Version 2.01- www.technelysium.com.au) and Vector NTi version 11 (Invitrogen, Carlsbad, CA) were used for all sequence and alignment analysis. The full-length sequence for Lu (NCBI database) has the accession number AF170726.

RESULTS AND DISCUSSION

Characterisation of myxoma virus isolates

Using the knowledge gained from the genetic typing study of myxoma viruses with known virulence grades (15) and the characterization of myxoma vaccine strains (unpublished data) we have used a combination of PCR and sequencing to characterize and distinguish virus strains currently circulating causing myxomatosis outbreaks on Spanish rabbit farms. We received samples from 22 rabbit farms throughout 2009 and 2010. The analysis of the sequence data from these samples has revealed that, in the regions of the genome that we have studied are highly conserved, with few differences between samples when compared to the Lu sequence. The main exception to this is a 21 nt deletion in the M009L gene observed in the majority of samples analyzed. The virus sequences obtained from samples were distinguishable from the vaccine strain sequences.

We based our studies on characterization of myxoma viruses on two different strategies, genomic and antigenic. In an attempt to differentiate virus strains antigenically we carried out preliminary Western blot studies of infected cell extracts using sera positive by ELISA for myxoma virus antibodies from a number of wild rabbits. Due to the large heterogeneity of the results observed between sera (data not shown) we decided to obtain sera from vaccinated rabbits using the three main commercially available vaccine strains. Regardless of the vaccine used, a large percentage of the animals vaccinated failed to generate anti-myxoma virus antibodies as measured by ELISA. Although the number of animals used in this experiment was relatively small the result prompted us to analyze sera and measure antibody levels in vaccinated animals from a larger number of commercial rabbitries. These rabbitries had no prior history of myxoma virus infections.

Analysis of myxoma virus vaccination strategies.

During 2011 we measured anti-myxoma virus antibody levels from 8 different rabbit farms (10 – 18 animals per farm) vaccinated subcutaneously with different homologous vaccines. The average data of seropositivity (IR>2 by ELISA) for these farms was between 20 and 40 %, confirming the results observed in the preliminary experiment. This suggested that a more profound study was required as to the causes for low numbers of seroconverters.

Using the same lot of homologous vaccine and two types of vaccine administration protocol (subcutaneous using needle or intradermic using Dermojet) we estimated the antibody response in vaccinated animals on 4 different rabbit farms that were free of myxomatosis. Between 10 and 24 animals were analyzed for each farm.

The overall results of this vaccination study are shown in Table 1. The levels of seropositivity (ELISA IR>2) indicate that in this study Dermojet was the favorable vaccine application method and that higher levels of seropositivity were achieved using two injection sites instead of one. A representative example of the results found in one of the experiments of vaccination is presented in Figure 1.

Table 1. Seropositivity (%) of vaccinated rabbits

Protocol	**% seropositivity (RI > 2) 1 month after vaccination**
Dermojet	100
A single Subcutaneous injection	25-80
Two Subcutaneous injections	90

Virulence grading and challenge

Using the sequence data we obtained from currently circulating (wild or) field strains we selected one representative sample for virus isolation and virulence characterization in rabbits. The virus isolate was termed Granada-05/09. In order to measure the virulence grade of the Granada-05/09 strain we carried out experimental infection of 6 rabbits in the level 2 biosecurity containment facility of the University of León. Three rabbits were inoculated intradermally and three subcutaneously. Five of the rabbits inoculated with the Granada-05/09 myxoma virus strain, died between 6 and 8 days post

infection, while the sixth rabbit (via intradermic inoculation) died 14 days post infection. Indicating that the currently circulating myxoma virus strain analyzed is of the maximum virulence grade (A).

We also tested the ability of this virus to infect previously vaccinated rabbits. A total of 12 rabbits were used in the challenge study. Six animals were vaccinated intradermally using a Dermojet and six animals were vaccinated subcutaneously using a needle. Sera were taken from animals pre-vaccination, 15 and 30 days post vaccination. One month post vaccination rabbits were challenged using the Granada-05/09 viral strain.

The 6 animals vaccinated using the Dermojet protocol were completely protected from challenge with the virulence grade A Granada-05/09 strain, showing no clinical signs of myxomatosis. These animals had high antibody levels one month post vaccination and these levels were maintained after challenge.

From the 6 animals vaccinated using needles 1 animal that was seronegative by ELISA 1 month post vaccination showed clinical signs of myxomatosis 7 days post challenge. Four animals that had low levels of seropositivity one month post vaccination were fully protected but levels of antibodies in sera rose substantially post challenge. The remaining animal had high levels of anti-myxoma virus antibodies 1 month post vaccination, these levels remained high post challenge and the animal showed no clinical signs of myxomatosis.

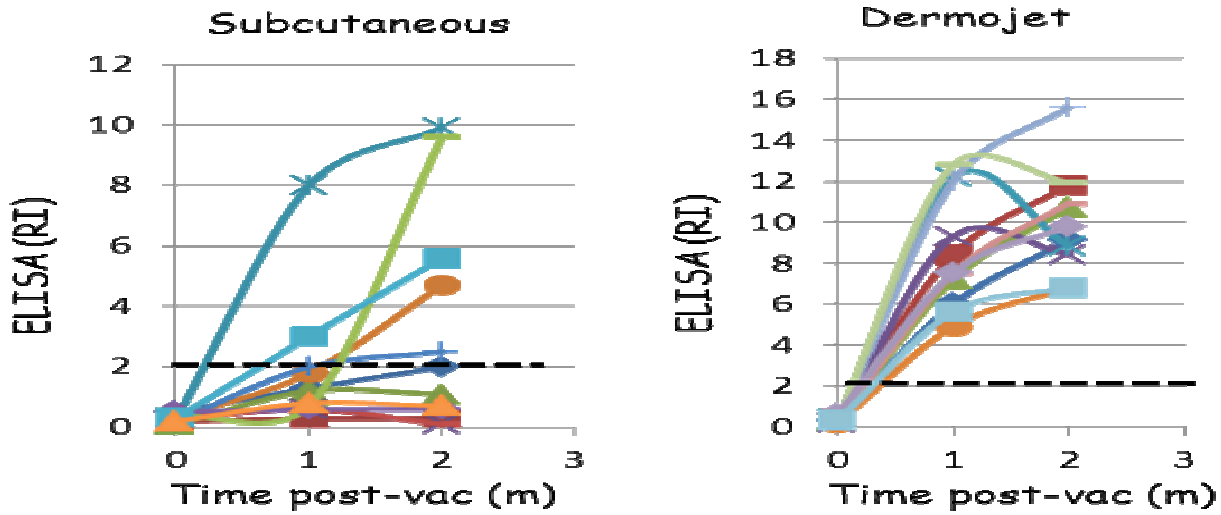

Figure 1: Detection of antibody response in rabbits vaccinated subcutaneously or intradermally using Dermojet. Post-vaccination time is shown in months. ELISA (RI) relative index >2 is considered seropositive as shown using dashed line.

Although a reduced number of animals have been used in this study the results from this challenge experiment indicate that the current vaccine can protect against this currently circulating virulent strain of myxoma virus. The study also shows that when administered via the subcutaneous route a percentage of animals remain seronegative and may not be fully protected from disease.

CONCLUSIONS

We have sequenced selected genes from myxoma virus isolates causing outbreaks of myxomatosis from 22 farms during 2009 -2010. A representative strain was selected, isolated and used in animal experiments and is of virulence grade A (maximum virulence). Challenge studies indicate that animals vaccinated with a current vaccine strain with high levels of antibodies are protected from challenge with the Granada-05/09 strain. Vaccination trials indicate that subcutaneous vaccination gives reduced numbers of seroconverted animals when compared to animals using an alternative route of vaccine administration (Dermojet).

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REFERENCES

Alda F, Gaitero T, Suarez M, Doadrio I: Molecular characterisation and recent evolution of myxoma virus in Spain. *Arch Virol* 2009.

Barcena J, Pages-Mante A, March R, Morales M, Ramirez MA, Sanchez-Vizcaino JM, Torres JM: Isolation of an attenuated myxoma virus field strain that can confer protection against myxomatosis on contacts of vaccinates. *Arch Virol* 2000, 145:759-771.

Cameron C, Hota-Mitchell S, Chen L, Barrett J, Cao JX, Macaulay C, Willer D, Evans D, McFadden G: The complete DNA sequence of myxoma virus. *Virology* 1999, 264:298-318.

Cavadini P, Botti G, Barbieri I., Lavazza A, Capucci L. Molecular characterization of SG33 and Borghi vaccines used against Myxomatosis. *Vaccine* 28 (2010) 5414–5420

Dalton KP, I Nicieza, A Baragaño, JM Martin Alonso and F. Parra. Molecular characterisation of virulence graded field isolates of myxoma virus. *Virology Journal* 2010, **7**:49 doi:10.1186/1743-422X-7-49.

Farsang A, L. Makranszki, M. Dobos-Kovacs, G. Virag, K. Fabian, T. Barna, G. Kulcsar, L. Kucsera and F. Vetesi, : Occurrence of atypical myxomatosis in Central Europe: clinical and virological examinations. *Acta Veterinaria Hungarica* 2003, 51:493-501.

Fenner F, Marshall ID: A comparison of the virulence for European rabbits (Oryctolagus cuniculus) of strains of myxoma virus recovered in the field in Australia, Europe and America. *J Hyg (Lond)* 1957, 55:149-191.

Guerin JL, Petit F., Van Es A., Gelfi J., Py R., Bertagnoli S., Boucraut-Baralon C. Analyse moléculaire des souches vaccinales SG33 et Poxlap du virus myxomateux: implications prophylactiques et épidémiologiques. 7émes Journ Rech. Cunicole Fr., Lyon, 1998

Kerr PJ, Merchant JC, Silvers L, Hood GM, Robinson AJ: Monitoring the spread of myxoma virus in rabbit Oryctolagus cuniculus populations on the southern tablelands of New South Wales, Australia. II. Selection of a strain of virus for release. *Epidemiol Infect* 2003, 130:123-133

Kritas SK, Dovas C, Fortomaris P, Petridou E, Farsang A, Koptopoulos G: A pathogenic myxoma virus in vaccinated and non-vaccinated commercial rabbits. *Res Vet Sci* 2008, 85:622-624.

Labudovic A, Perkins H, van Leeuwen B, Kerr P: Sequence mapping of the Californian MSW strain of Myxoma virus. *Arch Virol* 2004, 149:553-570.

Marlier D, Mainil J, Boucraut-Baralon C, Linden A, Vindevogel H. The efficacy of two vaccination schemes against experimental infection with a virulent amyxomatous or a virulent nodular myxoma virus strain. J Comp Pathol. 2000 Feb-Apr;122(2-3):115-22.

Marlier D, Herbots J, Detilleux J, Lemaire M, Thiry E, Vindevogel H: Cross-sectional study of the association between pathological conditions and myxoma-virus seroprevalence in intensive rabbit farms in Europe. *Prev Vet Med* 2001, 48:55-64.

Muller A, Silva E, Abrantes J, Esteves PJ, Ferreira PG, Carvalheira JC, Nowotny N, Thompson G: Partial sequencing of recent Portuguese myxoma virus field isolates exhibits a high degree of genetic stability. *Vet Microbiol* 2010, 140:161-166.

Saint KM, French N, Kerr P: Genetic variation in Australian isolates of myxoma virus: an evolutionary and epidemiological study. *Arch Virol* 2001, 146:1105-1123.

Zuniga MC: A pox on thee! Manipulation of the host immune system by myxoma virus and implications for viral-host co-adaptation. *Virus Res* 2002, 88:17-33.