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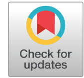
Highly efficient vaccines for Bluetongue virus and a related Orbivirus based on reverse genetics



Polly Roy

Bluetongue virus (BTV) reverse genetics (RG), available since 2007, has allowed the dissection of the virus replication cycle, including discovery of a primary replication stage. This information has allowed the generation of Entry-Competent-Replication-Abortive (ECRA) vaccines, which enter cells and complete primary replication but fail to complete the later stage. A series of vaccine trials in sheep and cattle either with a single ECRA serotype or a cocktail of multiple ECRA serotypes have demonstrated that these vaccines provide complete protection against virulent virus challenge without cross-serotype interference. Similarly, an RG system developed for the related African Horse Sickness virus, which causes high mortality in equids has provided AHSV ECRA vaccines that are protective in horses. ECRA vaccines were incapable of productive replication in animals despite being competent for cell entry. This technology allows rapid generation of emerging Orbivirus vaccines and offers immunogenicity and safety levels that surpass attenuated or recombinant routes.

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Introduction

Virus reverse genetics (RG) techniques are based on the ability to rescue viable virus containing designed genetic changes. The role of those changes in virus replication or pathogenicity can then be assessed. The technique relies on the transfection of cells with nucleic acids encoding the viral genome, or mutants thereof, and was developed first for DNA viruses and subsequently for positive-sense RNA viruses. Gradually reverse genetics technology has been developed for all the major virus families. The techniques have particularly become the method of choice for the design of efficacious and safe vaccines with specific changes in viral components added to induce strong protective immunity in the host without health risks or deleterious effects.

In contrast to the development of RG systems for positive and negative strand RNA viruses, the development of RG systems for double-stranded RNA (dsRNA) viruses of the *Reoviridae* family was relatively slow, with the first two *Reoviridae* RG systems, for Bluetongue virus (BTV) and reovirus, not having been developed until 2007 [1,2•]. They were, nevertheless, brought to fruition and in the last decade these systems have enabled remarkable progress in the understanding of the replication process and pathogenesis of these viruses. Further, RG systems have been used successfully to develop attenuated vaccine strains for 2 different Orbiviruses, BTV and the closely related African Horse sickness virus (AHSV).

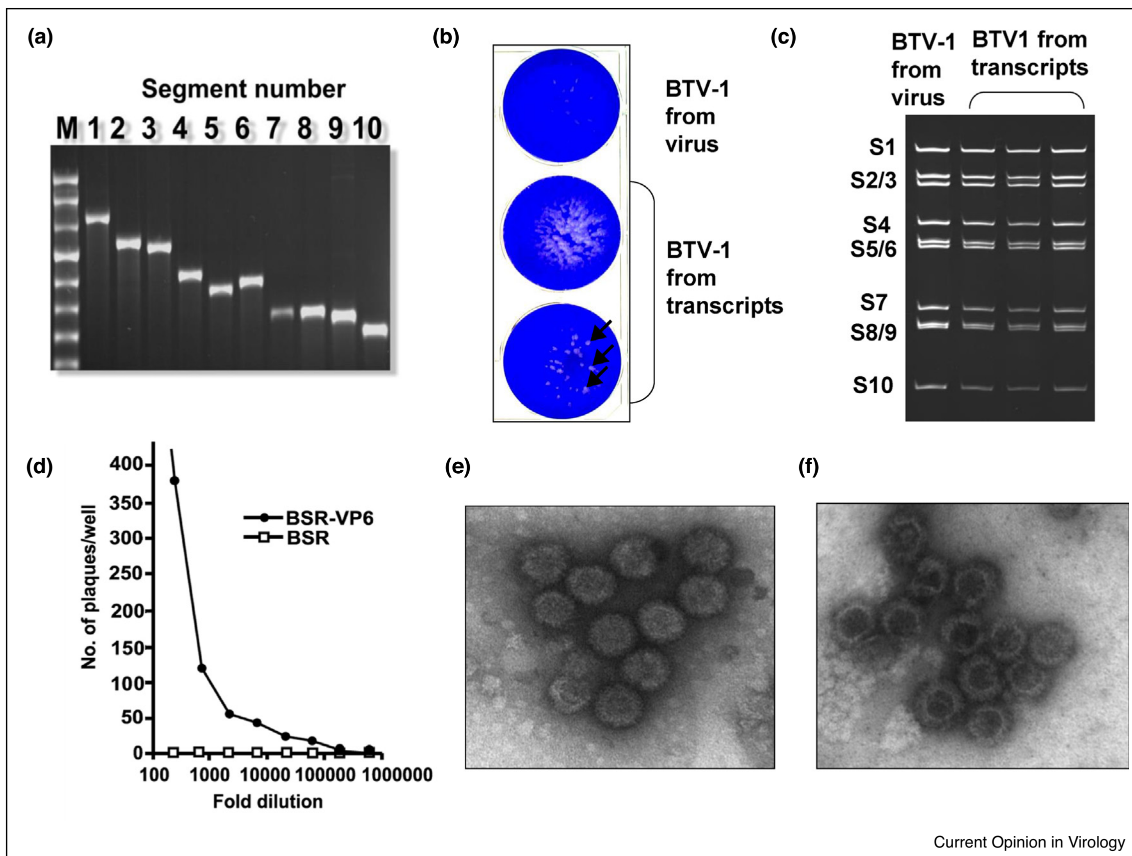
BTV and AHSV are insect-vectored emerging pathogens of wild and domestic animals which have a severe economic impact on the worldwide agricultural industry. BTV causes disease in ruminants, for example, sheep, goat, and cattle, with a mortality rate of up to 70% in certain breeds of animals. It is endemic in many tropical and subtropical countries and represents an ongoing threat to livestock in both developed and developing countries. AHSV is one of the most devastating diseases of horses characterized by high fever, respiratory distress, lethargy, and has an extremely high mortality rate (up to 90%). Both viruses are transmitted by the same insect vector, the *Culicoides* midge, and thus there is the potential for AHSV outbreaks anywhere in the world, similar to recent BTV outbreaks in Europe [3,4]. Current commercially available live virus vaccines against these viruses are considered neither safe nor sufficiently effective to be used widely. These issues are further compounded by the existence of multiple serotypes of these viruses, 27 for BTV, and 9 for AHSV. Therefore, rationally designed safe vaccine strains that protect against these viruses are highly desirable.

Bluetongue virus and development of revere genetics

The virion is a non-enveloped icosahedral particle composed of three concentric protein layers enclosing a segmented genome of 10 linear dsRNA molecules (S1-S10) (Reviewed [5,6]). The outercapsid, composed of two major structural proteins, VP2 and VP5, is involved in cell attachment and membrane penetration during the initial stages of infection [7–9]. The receptor attachment protein, VP2, is

the serotype determinant and is the most variable viral protein [10,11]. After entry, the outer layer of the virion particle is uncoated in the endosome prior to the release of the remaining core particle into the cytoplasm of the host cell [12]. The core particle is made up of two concentric protein layers, the surface VP7 layer and an underlying VP3 layer, which surrounds the genomic dsRNA segments together with the replicase complex of three enzymatic minor proteins, VP1, VP4, and VP6 [13,14]. While the viral genomic dsRNAs never leave the core particle, the core particle itself is transcriptionally active, synthesizing and extruding multiple capped single-stranded mRNA copies of each viral genome segment into the host cell cytoplasm [15,16]. These transcripts have the dual roles of encoding the 11 viral proteins (seven structural and four nonstructural) and serving as templates for the synthesis of the new viral dsRNA segments. As defined by the Baltimore classification scheme, all viruses, including complex viruses such as BTV replicate via the production of a unique set of viral transcripts and that viral single-stranded RNAs (ssRNAs), delivered to the host cell cytoplasm by transfection should allow both critical functions of virus RNA, translation and replication, to be achieved. Thus, to develop BTV RG, the possibility of recovering infectious BTV following the transfection of permissive cells with BTV transcripts (ssRNAs) was assessed. Transfection of 10 BTV transcripts synthesized *in vitro* using BTV core particles was indeed found to be sufficient to establish BTV infection in permissive cells [2•]. Subsequently, the recovery of BTV entirely from *in vitro* synthesized T7 transcripts, derived from cDNA clones, was investigated and found to be a viable means of generating BTV with a fully defined genome (Figure 1) [17]. This method permits the recovery of mutants in a consistent genetic background with no screening required to remove wild-type virus. The ability to generate specific mutants provides a robust tool not only to investigate the different stages of BTV replication cycle but also for the generation of specifically modified vaccines for BTV that are both non-transmissible and highly efficacious.

Figure 1



The reverse genetics system and its use to generate ECRA vaccines. **(a)** All 10 segments of the BTV genome are transcribed in vitro to include any genetic changes to be introduced into the virus. **(b)** Transfection of cells with all 10 transcripts results in virus recovery and plaque formation. **(c)** Characterization of the genomes of rescued viruses shows them to have a full complement of segments which match those of wild type virus. **(d)** The infectivity and VP6 helper cell line dependency of the ECRA virus. **(e)** Negative stain EM of wild type viral core particles. **(f)** Negative stain EM of ECRA viral core particles. Note the lack of a genome as a consequence of the deletion of VP6.

Designing novel BTV vaccines based on RG system

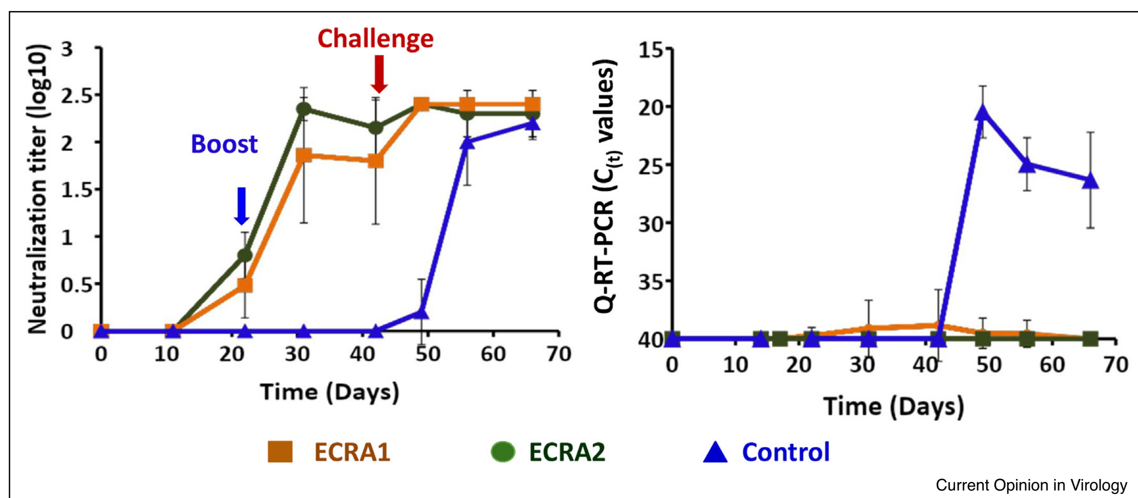
A modified virus recovered from cloned genes should facilitate the rapid generation of defined attenuated vaccine strains. One class of these are ECRA (Entry Competent Replication Abortive) viruses, formally known as 'DISC' viruses. These strains can enter BTV-permissive cells, disassemble, and synthesise viral proteins, but are incapable of replication due to the lack of an essential gene product. However, the modified strains still trigger an innate immune response and the subsequent development of neutralizing antibodies. Such viruses are incapable of producing infectious virions unless the deleted function is provided in *trans* by a complementing cell line. To this end, we targeted an essential component of primary replicase complex, VP6 which is responsible for the ssRNA packaging into the capsid and is believed to assist in unwinding the duplex RNAs [18,19•]. A series of BTV-1 mutant viruses targeting VP6 [18], were rescued by RG and then examined for their capacity to replicate both in wild-type cells and in a VP6-expressing cell line [19•]. Characterization of the growth properties of mutant viruses showed that each mutant had the necessary characteristics for a potential vaccine strain: (i) viral protein expression in non-complementing mammalian cells, (ii) no infectious virus generated in non-complementing cells, and (iii) efficient replication in the complementing VP6 cell line (Figure 1).

To assess VP6 RG viruses as vaccine candidates, two VP6-deletion mutant viruses, were used to vaccinate two groups of six sheep, each injected subcutaneously between $\sim 5 \times 10^6$ to 1×10^7 PFU and a group of animals was inoculated with VP6-complementing cell lysate as control [19•]. Animals were boosted with the same vaccine preparations or VP6-cell lysate 21 days after the first vaccination and blood and serum samples were collected at regular intervals to monitor virus replication and the immune response. All vaccinated animals seroconverted by 7 days post-vaccination, in contrast to the control group where no specific anti-BTV antibodies were detected. Further, all immunized animals produced a neutralizing response suggesting that the two VP6 deletion viruses were capable of inducing BTV antibody responses in the vaccinated animals (Figure 2). Whether the elicited antibodies were sufficient to protect the vaccinated sheep against virulent virus, was subsequently assessed by challenge with BTV-1 at 42 days post-vaccination and blood samples were taken at regular intervals. All samples were tested for both group-specific protein VP7 antibodies by ELISA using and for type-specific neutralizing antibody titres. Vaccinated animals displaced a sustained antibody response throughout the experiment by both ELISA and titre, whereas the control animals only developed group-specific and neutralizing antibodies after challenge. More importantly, BTV-specific quantitative real-time RT-PCR test of blood samples of each animal failed to detect any genomic RNA in vaccinated animals (Figure 2), confirming the absence of BTV replication throughout the vaccination trial study.

Rapid generation of a reassortant ECRA vaccine strain and its protective immune response in sheep

Since the BTV 'core' elements are conserved, the same core can be used for generating vaccines for new serotypes simply by substituting the outer capsid protein genes. Indeed, it was possible to rapidly generate a reassortant ECRA virus that consisted of the BTV-1 core with the outer capsid proteins, VP2 (serotype determinant) and VP5, of BTV-8 (BTV-1/8D1) a highly

Figure 2



Trial data of two ECRA vaccine candidates. **Left:** Neutralizing antibody titres following prime, boost and challenge with virulent BTV. **Right:** Viremia detected by RT-PCR of the BTV genome. The low level transient positivity from day 20 in the ECRA 1 sample is the input vaccine sample.

pathogenic serotype, by transfecting cells with the VP2 and VP5 encoding RNA segments of BTV 8, together with the remaining eight segments from the replication defective BTV1 [19•]. The protective capabilities of this reassortant BTV-8 ECRA virus were assessed in sheep by challenge with a virulent BTV-8 strain as described for the BTV-1 ECRA vaccine strain. The data demonstrated conclusively that this vaccine was highly protective and could offer a promising alternative to the currently available attenuated and killed virus vaccines. The vaccines are also DIVA compliant (differentiating infected from vaccinated animals) as the lack of VP6 is a distinguishing feature of the vaccine but not the true infection.

Protective efficacy of monovalent and multivalent BTV ECRA vaccine strains in sheep and cattle against virulent virus challenge

The success and ease of vaccine generation for BTV-1/8 strain was then extended by exchanging the two RNA segments responsible for serotype determination to generate a series of monovalent ECRA vaccine strains for different BTV serotypes, including the European serotypes that have caused serious disease in animals. Accordingly, reassortant ECRA viruses were generated for six different BTV serotypes (BTV-2, BTV-4, BTV-10, BTV-13, BTV-21, and BTV-24) by substituting segments S2 and S6 (encoding VP2 and VP5, respectively) and fully characterized as described above [20]. The immunogenicity of each of these was then assessed in cattle.

Naïve heifers (3 in each group) between 9 and 18 months of age were vaccinated with one of ECRA BTV-2, BTV-4, or BTV-8, in two doses administered 21 days apart. A further nine received a lysate of complementary cells as controls. Clinical score and rectal temperature were taken daily from 4 days before the first vaccination (V1) until the end of the challenge experiment (day 63). Serum samples were taken at regular intervals from day 4–21 days after both the first and second vaccinations. 42 days post vaccination, vaccinated animals and three controls were challenged by subcutaneous delivery of 4×10^5 $TCID_{50}$ of a virulent BTV strain of the homologous serotype. Whole-blood samples of post-challenged animals were taken at regular intervals and serum samples were collected at 0-days and 21-days post challenge. All vaccinated animals had neutralizing antibodies against homologous serotypes and virus replication was not detectable in the vaccinated animal samples post challenge, in contrast to the control animal samples.

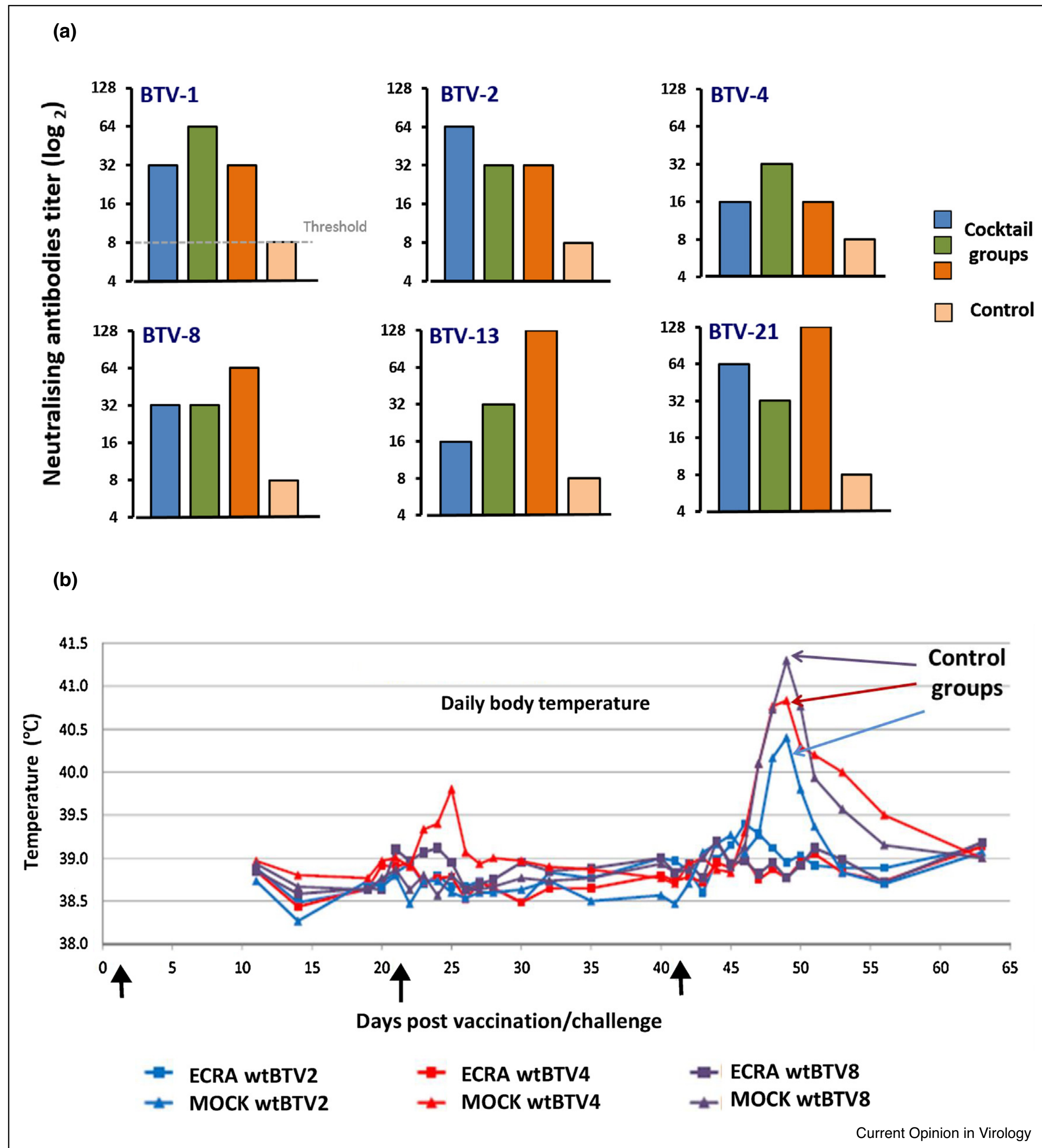
Simultaneous circulation of two or more serotypes of BTV emphasizes the necessity of a vaccine that protects against several serotypes. Working toward the development of a multiserotype vaccine, similar to the three polyvalent, live attenuated vaccines currently in use in South Africa, a cocktail mixture of six ECRA viruses (BTV-1, BTV-2, BTV-4, BTV-8, BTV-13 and BTV-21), all identified in European outbreaks [4], was also assessed [20]. Three groups of six sheep were inoculated with the cocktail vaccine ($\sim 1-4 \times 10^5$ PFU/strain) in a prime and boost protocol. One group of nine sheep received an equivalent dose of cell lysate and served as the control group. Blood samples were collected at regular intervals over the experimental period to determine the serological response to vaccination and for detection of virus replication. In parallel, body temperatures, as a sign of disease, were recorded routinely for all groups.

All animals vaccinated with the cocktail vaccine had neutralizing antibodies against all the serotypes included (BTV-1, BTV-2, BTV-4, BTV-8, BTV-13, and BTV-21) and there was no apparent interference with respect to the ability of the animal to respond to each of the vaccine strains present in the cocktail suggesting that, in practice, such a cocktail vaccine would be able to protect animals against all included serotypes. On day 42, each group of vaccinated sheep and three control sheep were challenged with virulent viruses, either BTV-2 or BTV-4 or BTV-8, and monitored for a further 21 days (Figure 3).

After challenge, none of the vaccinated animals had disease symptoms irrespective of the vaccine given, monovalent or multivalent, whereas all control animals showed severe clinical signs associated with bluetongue disease (Figure 3). Further vaccinated animals had no sign of virus replication, in contrast to all the control animals which showed virus replication as expected. Moreover, in a subsequent study, when groups of sheep were vaccinated with a cocktail of six different vaccines similarly and challenged at 21 days (early) or 154 days (5 months, late) post-vaccination with virulent viruses, all vaccinated animals, both in the early challenge or the late challenge group, were protected and had no signs of clinical disease. Protection was also achieved in cattle either with monovalent ECRA vaccine strains or a cocktail of three vaccine strains, albeit at a lesser level than in sheep.

To advance the ECRA vaccines to the marketplace, a number of parameters were tested for their effect on vaccine performance, such as the genetic stability of vaccine strains and the complementing cell line, and optimal storage conditions, including additives, temperature, and desiccation [21]. These are all necessary for the protective capacity to be maintained following synthesis at industrial scale. In compliance with the manufacturing requirements of vaccine formulations without antibiotics, we found that the complementing VP6 cell line maintained a constant level of VP6 expression even at high passage numbers and in the absence of the selective agent (puromycin) in the growth media. Altogether, our data showed that the ECRA vaccine strains are genetically stable, and that virus titre can be maintained by desiccation in the presence of stabilizing

Figure 3



Performance of an ECRA cocktail vaccine of 6 BTV strains as indicated. **(a)** Neutralizing antibody responses of animals. Each column represents the average titres of six animals of each test group or three animals of each control group to the six individual BTV strains as indicated (modified from Figure 5 of Celma *et al.* [20]). The threshold for the assay was 8. **(b)** Body temperature as a marker of clinical signs recorded in animal groups post challenge with BTV-2, or BTV-4 or BTV-8.

reagents. These features are essential for mass production and vaccine administration in the field as no cold chain for delivery and handling is needed.

In addition, the ease of effective ECRA vaccine development using the RG system will facilitate the inclusion of new serotypes in existing vaccination programs. In this manner, a very-rapid-response vaccine could be developed based on which serotypes are circulating in the population at the time of an outbreak.

A further type of live-attenuated BTV vaccine has also been described. In this, the BTV virus is rescued in the absence of the gene encoding non-structural proteins

NS3/NS3a which is involved in virus egress and host interaction. The result is an attenuated strain named a Disabled Infectious Single Animal (DISA) vaccine. However, unlike ECRA, DISA vaccines are capable of low-level replication leaving one the possibility of the reacquisition of NS3 type functions from the host or a concurrent virus infection. Nevertheless, vaccine studies with DISA vaccines have shown protection in vaccinated animals and an inability to replicate in the *Culicoides* vector, which would likely block animal transmission [22,23].

AHSV RG-based vaccines that protect horses from African horse sickness disease

AHSV which is transmitted by biting midges similar to BTV causes severe disease in equids, with 95% mortality in naïve horses. Therefore, the development of efficient vaccines could be extremely important to counter the major economic losses caused by AHSV outbreaks in the equine industry. To this end and using the experience for BTV RG vaccines as an exemplar, we established a highly efficient RG system for AHSV serotype 1 (AHSV1) and, subsequently, a VP6-defective AHSV1 strain in combination with in *trans* complementation of AHSV VP6 [24–26]. This was then used to generate ECRA viruses for all nine serotypes, which required the exchange of two to five RNA segments to achieve equivalent titres of particles. All reassortant ECRA viruses could be amplified and propagated to high titres in cells complemented with VP6 but were wholly unable to replicated in any other cells. Initially, their protective efficacy was established using a model animal, the type I interferon receptor (IFNAR)-knockout mouse. These data were positive and subsequently, one monovalent (ECRA.A4) vaccine strain of AHSV4 and multivalent cocktail (ECRA.A1/4/6/8) vaccine strains of AHSV1, 4, 6, and 8 were tested in ponies [27•]. Two groups of four ponies were inoculated with each vaccine, and two ponies with control lysate, and two weeks after the booster vaccination all animals were challenged with virulent AHSV4, the most pathogenic virus of the serotypes used. In contrast to the control animals, all vaccinated ponies were protected and did not develop severe clinical symptoms of AHS. Furthermore, the multivalent cocktail vaccinated ponies produced neutralizing antibodies against all serotypes present in the cocktail, and a foal born during the trial was healthy and had no viremia. These results validate the suitability of these ECRA strains as a new generation of vaccines for AHSV.

Two further vaccine trials were undertaken in ponies: (1) to measure the duration of the antibody response of ECRA vaccine strain for up to 6 months and (2) to determine if all nine vaccine strains could be segregated into prime and boost regimes for achieving protective neutralizing antibodies against all serotypes. For the duration study, a monovalent ECRA AHSV4 strain was used as both prime and boost vaccine and for the cocktail vaccines, ponies were vaccinated with AHSV 1/4/7/9 ECRA strains as prime and boosted with AHSV 2/3/5/6/8 at day 21. Neutralizing antibodies remained present in all monovalent vaccinated animals for a minimum 24 weeks, although antibody titres peaked immediately after receipt of the booster injection. For cocktail vaccine trials, animals developed neutralizing antibodies response to all 9 AHSV serotypes, demonstrating that a multivalent vaccine based on our ECRA AHSV strains can be administered without the risk of cross serotype interference.

Together, the data obtained from a broad range of orbivirus vaccines produced using RG systems have demonstrated that they are as safe as subunit vaccines and express viral proteins at the sites of viral infection in animal hosts in a similar manner to that seen with the natural viruses. Thus, the vaccines have a safety profile similar to inactivated vaccines but with the cost advantage and innate immunity triggering properties of attenuated vaccines. The ECRA vaccine strains are grown in mammalian cells in large scale and there is no need to purify or inactivate, both costly processes. It is important to note that the production of the ECRA vaccines can be undertaken at BSL-2 conditions, since no self-replication competent materials are involved at any stage. Such a process can dramatically decrease the cost in comparison to the killed vaccine, which requires the generation of high titres of pathogen in high containment laboratories with subsequent inactivation and additional quality control steps. Another advantage of the ECRA virus strains is that a single strain can be used as the backbone of other serotypes by reassortment, facilitating rapid development of highly efficacious vaccine strains. Moreover, as ECRA strains do not replicate fully in the animal host, only express viral proteins once, the possibility of reassortment or recombination with a co-infecting wild-type virus is very low. The data shows that they can be administered as a multivalent cocktail vaccine conferring protection against all homologous virus infections without cross serotype interference. Reverse genetics technology has thus allowed the rapid and reliable generation of vaccines against the threat of an outbreak of bluetongue disease and AHS disease, regardless of the serotype involved. In principle, any emerging serotype can be prepared rapidly from the genomic material of the emerging strain to produce a safe vaccine and a robust, protective immune response.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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