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Evidence Project Final Report

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Project identification

1. Defra Project code: SE: 0542

2. Project title:
   Vector-borne transmission pathways and serological diagnostics of Schmallenberg virus

3. Contractor organisation(s):
   The Pirbright Institute
   Ash Road
   Woking
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4. Total Defra project costs (agreed fixed price): £114 636

5. Project: start date 01/03/2012

   end date 31/08/2012

6. It is Defra's intention to publish this form.

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

Schmallenberg disease was first observed in dairy cattle during the summer of 2011 in North Rhine-Westphalia, through clinical signs in adult animals that included pyrexia, decreased milk production and diarrhoea. Pooled blood samples from affected individuals were initially screened using metagenomic methods which highlighted seven orthobunyavirus-specific sequences. Phylogenetic analyses of these sequences highlighted that this was a novel virus (named tentatively as Schmallenberg Virus: SBV) within the Simbu serogroup. Members of this serogroup have a wide global distribution (although they are not present in northern Europe) and are largely vector-borne. While some related viruses within this serogroup do have an impact upon human health, (e.g. Oropouche virus), the most closely related viruses to SBV (e.g. Shamonda virus: SHV), do not pose this risk and in the absence of any epidemiological evidence of human infection, zoonotic potential is considered to be very low to negligible.

Following initial identification of clinical disease in adult ruminants, it has become increasingly clear that the primary clinical impact of SBV infection lies in its affect upon the developing foetus in cattle and sheep. Subsequent to the observation of clinical signs in adults, SBV was isolated from new-born lambs in the Netherlands possessing malformations. It soon became clear that these malformations were associated with large quantities of SBV partly localised in the central nervous system of affected individuals. The appearance of these clinical signs has led to retrospective discovery of SBV transmission in Belgium, France, Italy, Luxembourg and the United Kingdom. Since this time SBV has been identified in a vast swathe of Europe as far north as Sweden and as far south as Sicily.

Prior to the initiation of this project, the inference that SBV is vector-borne was based upon 1) a cessation in observation of clinical signs in adult cattle and sheep in line with a seasonal transmission of the virus; 2) the similarities in the current distribution of cases between SBV and bluetongue virus; 3) the close phylogenetic relationships with other viruses that are vector-borne within the Simbu serogroup. The range and related ecology of putative vectors is at present unknown, however, given the ubiquity of *Culicoides* biting midges on farms in the region and the local abundance of mosquitoes occasionally reported, anecdotally these groups would appear to be strong candidates for playing a primary role in transmission of SBV. In our study we used a series of experiments to characterise transmission of SBV using colonies of *C. sonorensis* (a US vector that is highly competent for a range of arboviruses); *C. nubeculosus* (the only UK species of *Culicoides* currently in colony) and *Culex pipiens pipiens* (an extremely common and abundant UK mosquito species derived from two sites in 2011).

Initially, we demonstrated that SBV could replicate to transmissible levels in all vector colony lines using intrathoracic inoculation. Individuals were injected with a cell culture derived strain of SBV and then incubated for a suitable period for each line to allow full dissemination of the virus. Individuals were then dissected into head and thorax/abdomen and SBV was recovered from all of these and additionally saliva using two different methods. Cq values (a semi-quantitative measure of the levels of SBV RNA) were recorded for these individuals which were then used in later experiments during comparative assessment of dissemination. We then used an artificial membrane-based method to feed both *Culicoides* and mosquitoes on a blood meal/SBV mixture heated to 37°C to simulate a live host. Fed individuals of all species were then either processed immediately to characterise the quantity of SBV imbibed in the original meal or allowed a period of incubation (the extrinsic incubation period) to develop and disseminate the virus.

SBV was found to replicate and fully disseminate in all vector lines when intra-thoracically inoculated into each species. Greater levels of SBV RNA were found in the whole processed *Cx. pipiens* than in *Culicoides*, a consequence of their greater size. Significant levels of SBV RNA were recovered from the saliva of infected individuals both directly and using a novel FTA card system. Similarly, Cx. pipiens took larger initial blood-meals resulting in greater SBV RNA being detected at day 0 than in *Culicoides*. Following the extrinsic incubation period, however, a total of over 300 membrane-fed *Cx. pipiens* from three lines did not produce Cq values indicative of fully disseminated infections. In contrast, approximately 19% of *C. sonorensis* (n=304) and 7% of *C. nubeculosus* (n=150) developed fully disseminated infections. SBV RNA and infectious virus were both recovered from C. sonorensis providing the strongest evidence to date of biological transmission of SBV by the genus and also provides a model species for future studies.

Studies of the replication of SBV in a KC-*C. sonorensis* cell line were hampered by a lack of replication, despite the line being originally derived from the *C. sonorensis* used during competence trials. This is unusual as this cell line had previously been used successfully to define the thermal limits of replication in bluetongue virus (BTV) and African horse sickness viruses. Additional preliminary data on the extrinsic incubation period in *C. sonorensis* demonstrated that SBV did not differ substantially in temperature-related replication from BTV. This would appear to suggest that the rapid spread of SBV is not related to differences in replication ability between the two viruses. In addition, no convincing evidence was found to support transovarial transmission of SBV in *C. sonorensis*.

Finally, an assay for detection of SBV antibodies based on a competition/blocking ELISA format using recombinant expressed proteins and monoclonal antibodies was devised. Using these reagents, which have no potential for contamination with the original virus, the resulting assay has no disease security restrictions concerning the movement and sharing of reagents or the assay itself. Consequently the assay can be rapidly shared with colleagues in other laboratories.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Exchange).

1. Introduction

Schmallenberg disease was first observed in dairy cattle during the summer of 2011 in North Rhine-Westphalia, through clinical signs in adult animals that included pyrexia, decreased milk production and diarrhea. Pooled blood samples from affected individuals were initially screened using metagenomic methods which highlighted seven orthobunyavirus-specific sequences. Phylogenetic analyses of these sequences highlighted that this was a novel virus (named tentatively as Schmallenberg Virus: SBV) within the Simbu serogroup. Members of this serogroup have a wide global distribution (although they are not present in northern Europe) and are largely vector-borne. While some related viruses within this serogroup do have an impact upon human health, (e.g. Oropouche virus), the most closely related viruses to SBV (e.g. Shamonda virus: SHV), do not pose this risk and in the absence of any epidemiological evidence of human infection, zoonotic potential is considered to be very low to negligable.

Following initial identification of clinical disease in adult ruminants, it has become increasingly clear that the primary clinical impact of SBV infection lies in its affect upon the developing foetus in cattle and sheep. Subsequent to the observation of clinical signs in adults, SBV was isolated from newborn lambs in the Netherlands possessing malformations. It soon became clear that these malformations were associated with large quantities of SBV partly localised in the central nervous system of affected individuals. The appearance of these clinical signs has led to retrospective discovery of SBV transmission in Belgium, France, Italy, Luxembourg and the United Kingdom. At the time of writing 121 farms have been identified in the UK as containing infected livestock (8 positive cases in cattle and 113 in sheep), with none of these farms reporting that stock were imported. Cases have currently only been reported from areas at risk of *Culicoides*-borne incursion during Autumn/Winter 2011, as identified by Defra project: SE: 4211.

To date, the inference that SBV is vector-borne is based upon 1) a cessation in observation of clinical signs in adult cattle and sheep in line with a seasonal transmission of the virus; 2) the similarities in the current distribution of cases between SBV and bluetongue virus; 3) the close phylogenetic relationships with other viruses that are vector-borne within the Simbu serogroup. The range and related ecology of putative vectors is at present unknown, however, given the ubiquity of *Culicoides* biting midges on farms in the region and the local abundance of mosquitoes occassionally reported, anecdotally these groups would appear to be strong candidates for playing a primary role in transmission of SBV. IAH currently maintains unique colonies of these insects and hence is well placed to carry out initial screening of vector competence following the emergence of new arboviruses. Previous studies have characterised closely related viruses using this approach (e.g. Akabane virus vector competence in *C. sonorensis* and *C. nubeculosus*) and these studies remain among the most solid evidence that these viruses have a vector component.

In this project used successive studies to characterise transmission of SBV using colonies of *C. sonorensis* (a US vector that is highly competent for a range of arboviruses); *C. nubeculosus* (the only UK species of *Culicoides* currently in colony); *Culex pipiens pipiens* (an extremely common and abundant UK mosquito species derived from two sites in 2011) and *Culex modestus* (a recently re-discovered mosquito that is rare in the UK but has been implicated in transmission of West Nile Virus in France, again derived in 2011). Through carrying out studies of infection, dissemination and transmission of SBV in these potential vector species, we hope to produce data that is useful in policy response. Outputs will include and assessment of specificity of transmission of SBV according to vector group, replication of SBV under known temperature regimes and the potential for transovarial transmission of the virus in insect hosts. These studies complement others that are ongoing for additional vector-borne diseases at IAH and will allow comparative conclusions to be drawn with BTV in relation to spread and persistence of SBV.

2. Objectives

1. To carry out intrathoracic inoculation studies in one species of *Culicoides* and mosquito line that will characterize the ability of Schmallenberg virus (SBV) to replicate in the haemocoel of putative vector species and allow full dissemination to be defined.
2. To carry out membrane feeding assays that will allow assessment of infection, dissemination and transmission potential of SBV in three lines (two species) of mosquitoes and two species of *Culicoides*.
3. To conduct studies to estimate the extrinsic incubation period of SBV in putative vector species identified through (1) and (2) and compare to data already collected for BTV.
4. To conduct a preliminary investigation into transovarial transmission of SBV in putative vector species.
5. To produce a suitable high-throughput and sensitive assay for detection of SBV specific antibodies in animals previously infected by the virus.

Objective 1. To carry out intrathoracic inoculation studies in one species of *Culicoides* and mosquito line that will characterize the ability of Schmallenberg virus (SBV) to replicate in the

haemocoel of putative vector species and allow full dissemination to be defined.

Summary: The primary aim of this objective was to examine the ability of SBV to replicate in the haemocoel of putative vector species derived from colonies at The Pirbright Institute. Five colony Dipteran lines were used for these studies: *Culicoides sonorensis* and *C*. *nubeculosus*; biting midge species which has been used in a series of previous investigations of vector competence for arboviruses and three separate lines of *Culex pipiens pipiens*, a common UK mosquito species recently colonised at the Institute. Using intrathoracic inoculation of a BHK-21 passaged strain of SBV, replication and dissemination of the virus in individuals of both *C. sonorensis* and *Cx. pipiens* were shown to occur in at least some individuals of both lines. In *C. sonorensis*, individuals inoculated and then incubated at 25°C for 7 days demonstrated highly consistent increased levels of SBV RNA (lower C_q values) and full dissemination (detection of SBV in saliva), in the majority of dissected individuals. In addition, all four FTA cards exposed to groups of *C. sonorensis* at day 7 post-inoculation tested positive for SBV RNA, validating this technique for use with *Culicoides* for the first time. In *Cx. pipiens* these results were more equivocal. While peak SBV RNA levels in IT inoculated individuals exceeded those recorded in inoculated *C. sonorensis* in some individuals (due to substantially larger body size), the range of SBV replication found in *Cx. pipiens* was far wider perhaps reflecting barriers to dissemination and/or a more effective immune response. This wider range was also apparent in dissected individuals.

1.1: Methods

Virus strain and arthropods used

The SBV strain used was derived from an isolation made using a *C. sonorensis* embryonic cell line in Germany by the Friedrich Loeffler Institut (FLI) and then sent to The Pirbright Institute following a single passage in a baby hamster kidney-21 (BHK-21) cells. At The Pirbright Institute the virus was then passaged twice on a BHK-21 cell line for intrathoracic (IT) inoculation studies. This strain was then used for intra-thoracic inoculation experiments while an additional passage in the BHK-21 cell line was used to produce a substantial volume of SBV for membrane feeding experiments (stored in a series of vials at -80°C). All viruses were used at C_q values of 10-12 with infectivity on BHK-21 cells recorded by cytopathic effect as 5.0-5.5 $\log_{10}TCID_{50}$.

C. sonorensis used during studies were of the PIRB-s-3 strain, originally derived from the Sorona (AAA) line propagated in Denver, Colorado, USA, established originally in 1957 by RH Jones. This line had been used previously in studies of Akabane virus infection in addition to a series of other vector competence studies. The *C. nubeculosus* line originated from UK stock established by P Mellor and has been maintained at The Pirbright Institute since the early 1970's using standardized techniques in an identical fashion to *C. sonorensis*. Two anautogenous *Cx pipiens* lines were established at The Pirbright Institute during 2011 from larvae collected from container/pond habitats in Surrey (subsequently named 'Brookwood' and 'Caldbeck'). Larvae were maintained on guinea pig chow during development in washing up bowls covered with netting and placed in cages prior to emergence from pupae. Adults were then fed on a Hemotek membrane system and allowed to feed on a horse-blood meal through a parafilm membrane overnight. Mating appeared to occur facultatively under laboratory conditions. An additional strain of *Cx pipiens* originating from a colony maintained at Wageningen (termed 'Netherlands' in this report) was also used as an outlier population. A *Cx modestus* line, also established in 2011 at The Pirbright Institute, was initially trialled for use but was not productive enough to be used in vector competence trials and discontinued in late August 2012.

IT inoculation studies were conducted with the *C. sonorensis* and *Cx pipiens* (Brookwood) lines. Approximately 300 *C. sonorensis* were lightly anaesthetized with CO_2 and then IT inoculated with 0.2µl of SBV using pulled glass capillary needles (Narishige, Japan) and a micro-injector equipped with a foot driver (Drummond Scientific Nanoject II: Drummond Scientific, USA). Ten IT inoculated *C. sonorensis* were processed immediately by sqPCR and the remainder incubated for 10 days at 25 ± 1°C with access to 10% sucrose solution. At day 10, surviving *C*. *sonorensis* were exposed in two groups to FTA® cards baited with Manuka honey using a previously described technique for detection of arboviruses in the saliva of mosquitoes. *C.sonorensis* were then immobilized using CO_2 and fixed to a piece of masking tape with their ventral surface exposed. A drop of pillocarpine (parasympathomimetic alkaloid: Sigma Aldrich, UK) solution (1:4 diluted) was then applied to the ventral surface of each *C. sonorensis* and saliva collected into a 1 µl microcapillary glass tube containing 10% FBS Glasgow's media (supplemented with 1000 IU/ml Penicillin/Streptomycin and 4µg/ml Amphotericin B). The collected media was then expelled into individual eppendorf tubes containing 0.5ml of Schneider's *Drosophila* Media (Gibco™) containing 10% foetal bovine serum (FBS). These final solutions were then stored at +4ºC prior to analysis. Approximately 200 *Cx pipiens* were IT inoculated using the same technique as in *C. sonorensis* but with 0.4µl of SBV. Ten IT inoculated *Cx pipiens* were processed immediately by sqPCR and the remainder incubated for 14 days at 25 ± 1°C with access to 10% sucrose solution. This extended period of incubation was used in agreement with

previous studies of vector competence in *Cx pipiens*. At day 14 mosquitoes were exposed in four groups to the FTA® cards as for *C. sonorensis*. Saliva was additionally collected as for *C. sonorensis* but the exposure period was extended to take account of previous studies.

The ten *C. sonorensis* tested for presence of SBV in saliva were subsequently decapitated using sterile needles (Monoject™ hypodermic needle, 18g x 1.5: Covidien, USA). Heads were ground in 100µl of SIM containing 1000 IU/ml Penicillin/ Streptomycin and 4µg/ml Amphotericin B using two coverslips. The remaining abdomen and thorax of each individual were also homogenized for 1 min at 25hz in 100µl of SIM using a TissueLyser® (Qiagen, UK) and 3mm stainless steel beads (Dejay Distribution Ltd., UK). In addition, a further 38 surviving *C. sonorensis* were homogenized as whole insects in 100 µl of SDM using the TissueLyser® system. Similarly, ten *Cx pipiens* were dissected and tested as for *C. sonorensis* and a further 35 individuals tested as whole insects.

Detection of Schmallenberg virus

Nucleic acid extraction was carried out using a Universal Biorobot (Qiagen, UK) in a 96-well format using a QIAamp® All Nucleic Acid MDx Kit (Qiagen, UK). SBV RNA in *Culicoides* samples was quantified using a sqPCR devised by the FLI that targeted the S segment of the genome. Duplicate assays were conducted from each extraction for the *C. sonorensis* studies only. In addition, infectious virus was isolated and quantified from selected samples using serial dilution and blind passage on BHK-21 cells. Presence of infectious virus was subsequently confirmed using observation of cytopathic effect at days 3 and 5 post-inoculation and by the sqPCR assay.

1.2: Results

IT inoculation led to fully disseminated SBV infections in all (10 out of 10) *C. sonorensis* examined (characterized by recovery of SBV RNA from the abdomen/thorax and the head). In the *Cx. pipiens* line (Brookwood), similar C_q values were recorded for the head and abdomen/thorax (Figure 1) to *C. sonorensis*. SBV quantity in saliva varied substantially in *Cx pipiens*, however, with a single individual yielding duplicate C_q values of 28.56 and 28.04 in comparison to maximum values of 31.56 and 30.86 in *C. sonorensis*. This could in part be due to the presence of salivary gland barriers to SBV dissemination in mosquitoes.

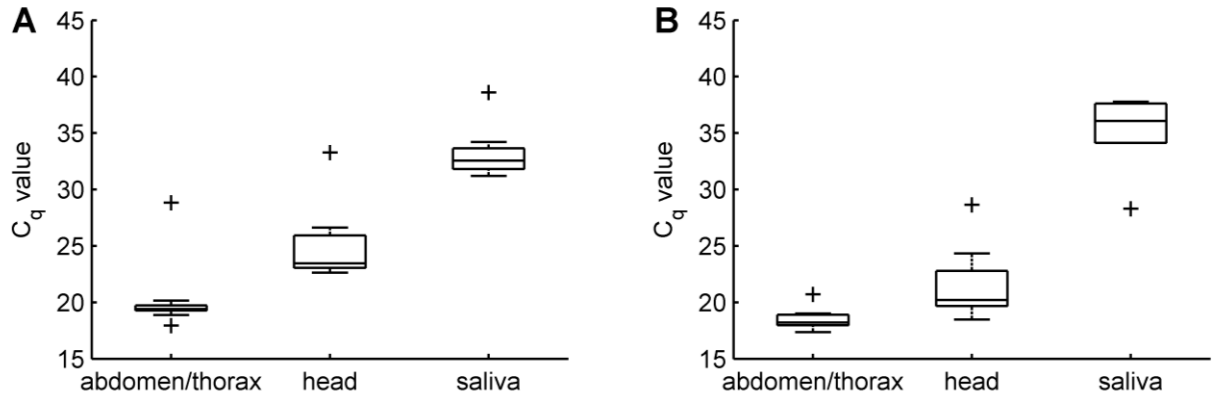


Figure 1. (A) C. sonorensis *infected via intrathoracic inoculation, incubated for ten days after which the abdomen/thorax, head and saliva of individual insects were processed separately using sqPCR. (B)* Cx pipiens *(Brookwood) infected via intrathoracic inoculation, incubated for fourteen days after which the abdomen/thorax, head and saliva of individual insects were processed separately using sqPCR. The box-and-whisker plot shows the median (horizontal line), interquartile range (box), 1.5 times the interquartile range (whiskers) and any outliers (crosses).*

Initial quantities of SBV RNA were greater in IT inoculated *Cx pipiens* (Brookwood) (mean C_q = 21.7 ±0.2 95% CL) than in *C. sonorensis* (mean C_q = 25.6 ±0.6 95% CL), due to the larger inoculum used and the larger size of the mosquito aiding accuracy of delivery. At day ten (*C. sonorensis*) and day fourteen (*Cx. pipiens*) post- inoculation, however, mean levels of SBV RNA in individuals were found to be similar (*Cx. pipiens*: mean C_q = 18.2 ±0.9 95% CL; range: 15.72-30.09; *C. sonorensis*: mean C_q = 19.3 ±0.1; range: 17.41-20.21). The increased variation in final quantity of SBV RNA in *Cx pipiens* could be due to barriers to dissemination of the virus that respond differently to *Culicoides* or to variation in immunological response. The FTA® card technique yielded at least one positive C_q value for both groups (of approximately 20 individuals each) of IT inoculated *C. sonorensis* (card 1: 34.21/34.73; card 2: 35.82/no C_q). In *Cx pipiens*,

individuals were exposed in four groups of approximately 10 individuals and only one SBV positive sample was produced (33.3/33.28). This indicated that the FTA cards would be unlikely to detect a single infected individual taking a sugar meal.

Objective 2. To carry out membrane feeding assays that will allow assessment of infection, dissemination and transmission potential of SBV in three lines (two species) of mosquitoes and two species of *Culicoides*.

Summary: The primary aim of this objective was to conduct infection studies of arthropods with SBV using a membrane-based feeding system to act as a proxy for viraemic ruminants. The arthropods used were fed on a defibrinated sheep-blood/SBV mixture and methods were applied to detect replication and dissemination of SBV in individual insects. *C. sonorensis* was demonstrated to be fully competent for SBV transmission including recovery of SBV from saliva elicited from individuals. The rate of competence of *C. sonorensis* for SBV was estimated at 11-19% for the strain used, providing a suitable model for further study. Competence of *C. nubeculosus* for SBV was found to be significantly lower than that of *C. sonorensis* in agreement with previous vector competence studies involving AKAV and these lines. None of the mosquito lines showed evidence of disseminated infections with only limited replication of SBV in all three lines. These results are used to interpret field studies of SBV infection in field collected *Culicoides* in the EU to date which suggest a wide range of competent vectors.

2.1: Methods

Batches of approximately 300-400, 2-3 day old adult *C. sonorensis* and *C. nubeculosus* were allowed to feed on a defibrinated sheep-blood (TCS Biosciences, UK) / SBV suspension via the Hemotek system (Hemotek Ltd, UK), using a Parafilm® membrane (Cole-Parmer, UK). Ten membrane fed *C. sonorensis* and *C. nubeculosus* were processed immediately for SBV RNA and the remainder incubated for 10 days at 25 ±1°C with access to 10% sucrose solution. Following incubation, 19 *C. sonorensis* and 20 *C. nubeculosus* were dissected as previously described for IT inoculated individuals. A further 304 *C. sonorensis* and 150 *C. nubeculosus* were also homogenized as whole insects. As an additional assessment of the presence of infectious SBV in incubated *C. sonorensis*, 30 individuals were fed and then selected following 10 days incubation at 25^0C, dissected and processed as for IT inoculated individuals. Homogenates of heads and abdomen/thorax from individuals with what were thought to be fully disseminated infections were inoculated onto BHK-21 monolayers in $25cm^2$ flasks containing 10ml of SDM and assessed by observation of cytopathic effect at days 1,2,3 and 4 post-inoculation. RNA was quantified in flasks immediately following inoculation using sqPCR and then at 4 days postinoculation. Replication was assessed from the appearance of cytopathic effect in samples and by comparison of the initial and final sqPCR Cq values. Confirmation of virus presence in the saliva of orally infected *C. sonorensis* at day 10 post infection was also assessed as described above for intrathoracic infected *Culicoides*. Four groups of approximately 70 orally infected *C. sonorensis* each were allowed to feed on FTA® cards baited with Manuka honey and card processed as previously described.

Three lines of *Cx pipiens* were tested for competence using the membrane feeding method (Brookwood; Caldbeck; Netherlands). Techniques were identical to those used with *C. sonorensis* with the exception of the following modifications. *Cx pipiens* lines were fed overnight on the membrane feeding device and at a reduced light intensity (as for colony production). The incubation period for replication of the virus was again extended to 14 days as for IT inoculation studies. SBV/blood mixtures used during these trials showed no apparent loss of infectivity during this period. Groups used during feeding were additionally smaller (due to the fact that the colonies involved were not as productive as those of *C. sonorensis* and *C. nubeculosus*), hence several batches of each line were fed sequentially. FTA® cards were not used due to variable results with IT inoculated individuals. Initial experiments were impeded by extremely high rates of mortality (≥95%) in the incubation period for all three strains, possibly due to fungal contamination of the storage pots during the extended holding period used. This was later overcome by transferring the mosquitoes to new pots at 7-10 days post-feeding. The number of each line of *Cx pipiens* processed for each treatment were as follows: Day 0 blood fed (Brookwood = 5; Caldbeck = 3; Dutch = 0); Day 14 incubated (Brookwood = 114; Caldbeck = 85; Netherlands = 122); Day 14 dissected (Brookwood = 17; Caldbeck = 18).

Statistical Analyses

To compare C_q values of dissected, IT inoculated *C. sonorensis* a linear mixed model was used with C_q value as the dependent variable, body component (abdomen/thorax, head or saliva) as a fixed effect and individual as a random effect. The methods were implemented using the nlme package in R. The C_q values obtained when *Culicoides* were infected by membrane feeding and processed as whole insects were analysed using a two-component mixture model. In this approach we assume that the C_q values for *Culicoides* with transmissible infections are drawn from one distribution, while those with sub-transmissible infections are drawn from another distribution. Based on these distributions we can assign each *Culicoides*

to either the "transmissible" or "subtransmissible" group with a certain probability based on its C_q value and estimate the proportion of *Culicoides* with a transmissible infection. Importantly, this avoids the need to use a potentially arbitrary threshold to define transmissible and sub-transmissible infections.

More formally, we assume the C_q values for transmissible and sub-transmissible infections are drawn from normal distributions with different means and standard deviations. In this case, the normally-distributed Cq value for a *Culicoides* is conditional on its Bernoulli-distributed (and unobserved) infection status, so that:

$$C_j \mid I_j \sim \mathrm{N}(\mu_{I_j}, \sigma^2_{I_j}),$$
$$I_j \sim \mathrm{Bern}(\phi),$$

where C_j is the observed C_q value for the jth *Culicoides*, I_j is the (unobserved) status of *Culicoides j* (i.e. transmissible (I_j=1) or sub-transmissible (I_j=0) infection), μ_i and σ_i are the mean and standard deviation of the C_q value for *Culicoides* of status i, respectively, and ϕ is the probability of developing a transmissible infection (i.e. competence). From this Bayes's Rule can be used to compute the probability that a *Culicoides* has a transmissible infection given its C_q value, so that:

$$\Pr(I=1 \mid C) = \frac{\phi f(C \mid \mu_1, \sigma_1)}{(1-\phi) f_0(C \mid \mu_0, \sigma_0) + \phi f(C \mid \mu_1, \sigma_1)},$$

where f is the probability density function (PDF) for the normal distribution and f_0 is the PDF for the normal distribution "zero-inflated" to incorporate observations with no C_q value, so that:

$$f_0(C \mid \mu_0, \sigma_0) = \begin{cases} p_0 & \text{no } C_q \text{ value}, \\ (1-p_0) f(C \mid \mu_0, \sigma_0) & \text{otherwise}, \end{cases}$$

where p_0 is the probability of no C_q value.

The mixture model (1) was implemented in a Bayesian framework, which requires a likelihood function and a joint prior distribution for the parameters. For the two-component mixture model, the likelihood for the data is:

$$L(\mathbf{I}, \mathbf{C} \mid \boldsymbol{\theta}) = \prod_j \left(\phi f(C_j \mid \mu_1, \sigma_1^2) \right)^{I_j} \left((1-\phi) f_0(C_j \mid \mu_0, \sigma_0^2) \right)^{1-I_j},$$

where f is the probability density function (PDF) for the normal distribution (with mean μ_i and standard deviation σ_i), $\boldsymbol{\theta}=\{\phi,\mu_0,\mu_1,\sigma_0,\sigma_1,p_0\}$ is a vector of parameters, **I** is a vector indicating the (unobserved) status of each *Culicoides* and **C** is a vector of observed C_q values. To ensure that the parameters in the model are identifiable, the mean C_q values for *Culicoides* with transmissible infections was constrained to be lower than the mean for *Culicoides* with subtransmissible infections (i.e. $\mu_1<\mu_0$).

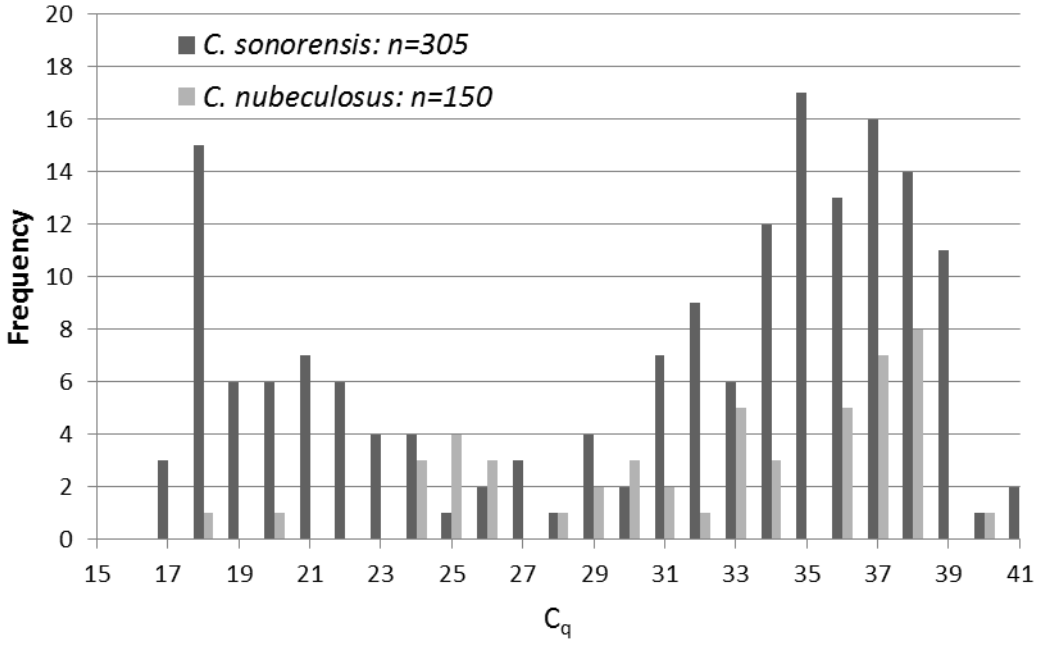
Non-informative priors were used for all parameters: Uniform(0,1) or diffuse exponential with mean 100, as appropriate. The only exception was the mean and standard deviation for C_q values in *Culicoides* in with a transmissible infection (μ_1 and σ_1) for *C. nubeculosus*, where informative priors were necessary for the methods to converge. Priors for these two parameters were constructed using the data on C_q values in *Culicoides* infected via membrane feeding and tested on day 0, which were assumed to reflect the C_q values that would be observed in *Culicoides* with a transmissible infection. A normal prior was used for μ_1 with mean equal to the estimated mean (24.24) and standard deviation (0.70) chosen so that 50% of the prior covered the 95% confidence interval. An exponential prior was used for σ_1 with mean equal to the estimated standard deviation (0.66). The priors were assumed to be independent of one another.

Parameter estimation was implemented in OpenBUGS (version 3.2.2; www.openbugs.info). Two chains, each of 200,000 iterations, were run, with the first 50,000 iterations discarded to allow for burn-in of the chain. The chains were then thinned (taking every twentieth iteration) to reduce autocorrelation amongst the samples. Convergence of the MCMC scheme was assessed visually and using the Gelman-Rubin statistic in OpenBUGS. Posterior predictive checking was used to assess model fit. More specifically, the posterior predictive distribution was used to generate replicated data by sampling parameter sets from the joint posterior distribution and using the sampled parameters to simulate data-sets using the above model for the C_q values. If the observed data generate a more extreme value of the measures than the replicate data (i.e. lie outside the 95% prediction interval), this provides an indication that the model does not adequately capture the data. In this case, histograms of the observed and simulated data were compared (with simulated C_q values above the maximum observed C_q value classified as giving no C_q value). In addition, the posterior predictive distribution was used to explore whether or not the probability of having a transmissible infection and the distribution of C_q values in *Culicoides* with a transmissible infection inferred from processing whole insects were consistent with the results for dissected insects, both intrathoracically inoculated and orally infected (i.e. lie within the 95% prediction interval).

2.2: Results

For the *C. sonorensis* processed as whole insects the observed C_q values showed a clear bimodal distribution. The results for *C. nubeculosus* and the *Cx. pipiens* were more equivocal with no such bimodal distribution in C_q values in individuals observed (Figure 2a,b).

2a)



2b)

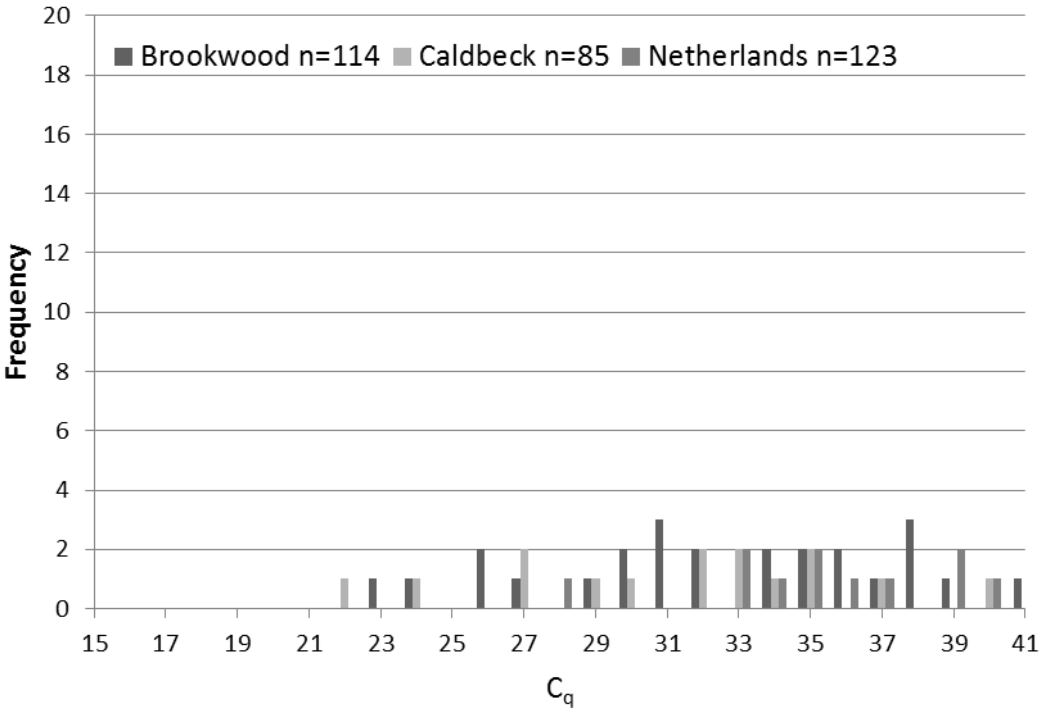


Figure 2. (A) Frequency distribution of C_q values produced from C. sonorensis *and C. nubeculosus infected via oral feeding with blood/SBV mixtures and incubated for ten days. Individuals were processed as whole insects using sqPCR. (B)* Cx pipiens *lines infected via oral feeding with blood/SBV mixtures and incubated for fourteen days.*

In individuals processed on the day of feeding, C_q values ranged from 22.0 ±1.1 in *Cx pipiens* to 22.3 ±0.2 in *C. sonorensis* and 24.2± 0.4 for *C. nubeculosus*. Only *C. sonorensis* and *C. nubeculosus* produced infections with lower C_q's than the upper confidence limit indicating unequivocally the presence of replicating SBV RNA. None of the C_q's produced by *Cx pipiens* individuals reached levels comparable with IT inoculated individuals and hence were excluded from further analysis. Of the dissected, orally infected insects, one (out of 19) *C. sonorensis* and one (out of 20) *C. nubeculosus* contained a fully disseminated infection. In *C. sonorensis* the C_q values (duplicate samples) for the abdomen/thorax, head and saliva were 18.98/19.20, 21.29/21.76 and 31.63/31.93, respectively. In *C. nubeculosus*, the C_q values (single samples)

were 18.20, 21.52 and 34.24, respectively. None of the *Cx pipiens* dissected demonstrated SBV RNA presence in all body parts indicative of a fully disseminated infection. The mixture model provided an acceptable fit to the observed C_q values. The probability of developing a transmissible infection (i.e. competence) was estimated to be 0.15 for *C. sonorensis* and 0.07 for *C. nubeculosus* (Table 1) although data for the latter species was less robust due to the smaller sample size used.

Parameter	mean	Median	95% CI*
C. sonorensis			
Probability of developing a transmissible infection	0.15	0.15	(0.11–0.19)
Mean C_q value			
transmissible infection	19.38	19.39	(19.06–19.71)
sub-transmissible infection	34.79	34.78	(33.99–35.64)
Standard deviation of C_q value			
transmissible infection	1.53	1.49	(1.21–2.11)
sub-transmissible infection	4.06	4.08	(3.27–4.71)
Probability of no C_q value in a sub-transmissible infection	0.51	0.51	(0.45–0.57)
C. nubeculosus†			
Probability of developing a transmissible infection	0.07	0.07	$(7.5\times10^{-4}$–0.15)
Mean C_q value			
transmissible infection	24.53	24.54	(22.82–25.56)
sub-transmissible infection	34.48	34.94	(31.51–36.56)
Standard deviation of C_q value			
transmissible infection	1.62	1.93	(0.03–3.41)
sub-transmissible infection	4.02	3.47	(2.27-6.49)
Probability of no C_q value in a sub-transmissible infection	0.71	0.71	(0.61-0.79)

* CI: credible interval
† N.B. The posterior distribution for *C. nubeculosus* is bimodal and the summary statistics must be treated with caution

The results of the mixture model (based on whole-insect processing) are comparable with the results for dissected *C. sonorensis* (both intrathoracically inoculated and orally infected). In particular, the number of *C. sonorensis* orally infected which developed a fully disseminated infection is consistent with probability of developing a transmissible infection estimated from the mixture model (Figure 3). Similarly, the C_q values observed in the abdomen/thorax and the head for those *C. sonorensis* infected via either route with fully disseminated infections are within the range expected for a transmissible infection. This was reflected in the parameter estimates for the mixture model, where the posterior distribution was bimodal.

The competence of *C. nubeculosus* was found to be significantly (P<0.001) lower than that for *C. sonorensis*. The mixture model provided an acceptable fit to the data. In addition, the number of *Culicoides* orally infected which develop a fully disseminated infection is consistent with probability of developing a transmissible infection predicted by the mixture model. The C_q values predicted by the mixture model, however, are higher than for that observed in the abdomen/thorax and the head for the one *Culicoides* with a fully disseminated infection. Using the mixture model, specifically equation (2), it is possible to infer the status of a *Culicoides* based on its C_q value. For *C. sonorensis* a C_q value below 24 implies a midge will have a transmissible infection, while a C_q value above 32 implies a sub-transmissible infection. For intermediate C_q values (i.e. between 24 and 32), the probability that a midge has a transmissible infection decreases from one to zero, but a particular individual could be in either class. The equivalent curve for *C. nubeculosus*, indicates that midges with a C_q value above 34 have a sub-transmissible infection, but the equivocal results for the mixture model make it difficult to discriminate between insects with transmissible infections from those with sub-transmissible infections at lower C_q values.

In orally infected *C. sonorensis*, all four groups of approximately 70 *C. sonorensis* tested also produced repeatable positive C_q values using this technique (FTA® card 1: 34.18/34.23; card 2: 35.34/35.49; card 3: 36.46/35.52; and card 4:36.47/38.46).Of the dissected, orally infected insects, one (out of 19) *C. sonorensis*

and one (out of 20) *C. nubeculosus* contained a fully disseminated infection. In *C. sonorensis* the C_q values (duplicate samples) for the abdomen/thorax, head and saliva were 18.98/19.20, 21.29/21.76 and 31.63/31.93, respectively. In *C. nubeculosus*, the C_q values (single samples) were 18.20, 21.52 and 34.24, respectively.

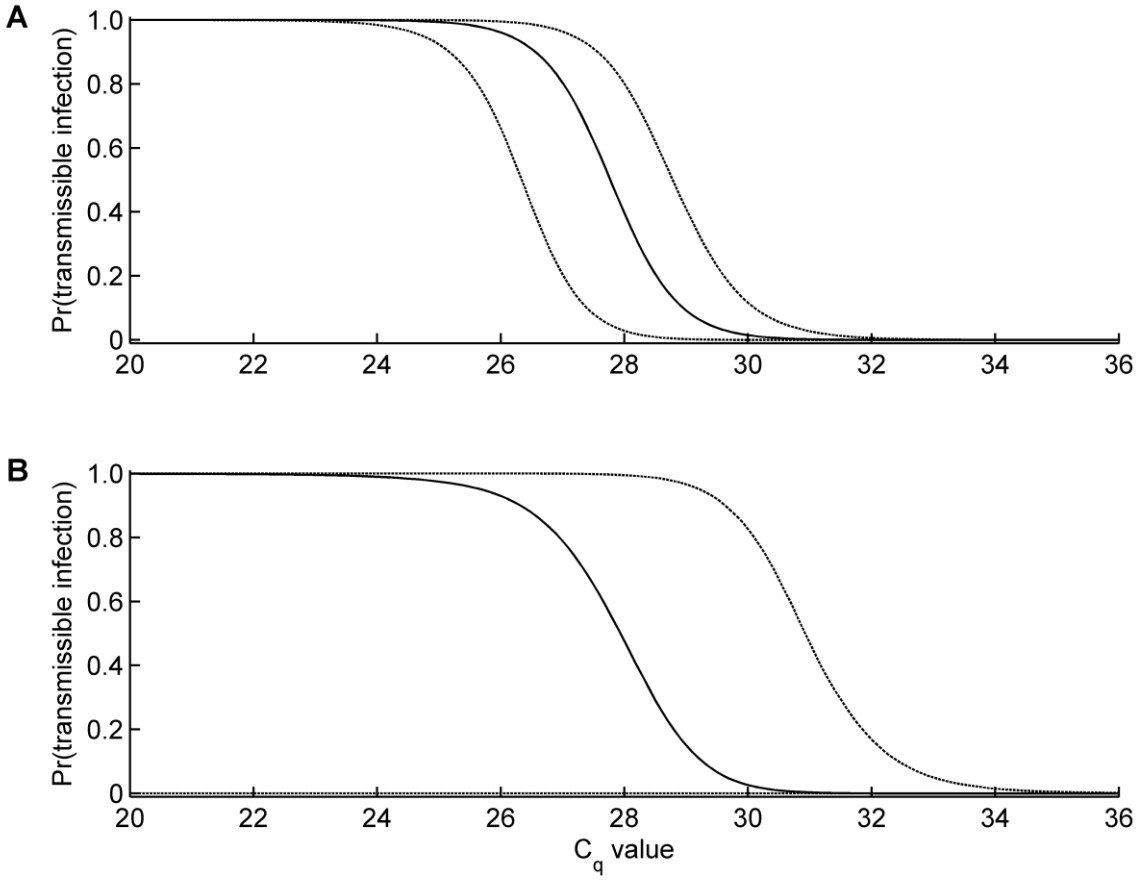


Figure 3. Inferred probability that a (A) C. sonorensis *or (B) C. nubeculosus with a given C_q value has a transmissible infection (derived using equation* ***Error! Reference source not found.****). The solid line is the posterior median and the dashed lines indicate the 95% credible interval*.

Using the mixture model it is possible to infer the status of a *Culicoides* based on its C_q value. For *C. sonorensis* a C_q value below 24 implies a midge will have a transmissible infection, while a C_q value above 32 implies a sub-transmissible infection (Figure 2A). For intermediate C_q values (i.e. between 24 and 32), the probability that a midge has a transmissible infection decreases from one to zero, but a particular individual could be in either class (Figure 3A). The equivalent curve for *C. nubeculosus*, indicates that midges with a C_q value above 34 have a sub-transmissible infection, but the equivocal results for the mixture model make it difficult to discriminate between insects with transmissible infections from those with sub-transmissible infections at lower C_q values (Figure 3B).

Using the data from this project, recent studies that have been conducted in the field indicate that the probability of *C. obsoletus*, *C. scoticus* and *C. chiopterus* in the Netherlands transmitting SBV was high as C_q values recorded using the same assay were very similar to those produced for *C. sonorensis*. In a separate study conducted in Belgium, far higher C_q values were provided from pools of heads, perhaps because, unlike the study in the Netherlands, an unoptimised homogenization step was used that could have reduced the levels of SBV RNA in samples. The rates of detection recorded in these studies were noticeably higher than those recorded for the BTV-8 outbreak in northern Europe, indicating the potential for vector competence for the SBV strain to be significantly greater than for BTV. This would agree with studies in Australia using the related AKAV, where transmission by *Culicoides* was found to be highly efficient. The species implicated in the Netherlands are abundant at farms across the UK and restriction of SBV spread in this region according to absence of vectors is highly unlikely. In addition, due to this high rate of transmission, control techniques applied at a farm level are likely to be less effective than found for BTV.

Objective Three: To conduct studies to estimate the extrinsic incubation period of SBV in putative vector species identified through (1) and (2) and compare to data already collected for BTV.

Summary: Attempts were made to carry out investigations of the EIP in both the KC-*C. sonorensis* cell line and *C. sonorensis* individuals identified as being competent for infection in Objective Two. Surprisingly, SBV was found to replicate extremely poorly in the KC-*C. sonorensis* cell line, precluding examination of low temperature replication of the SBV strain. In addition, FTA-card based techniques that were trialled as part of Objectives 1 and 2 failed to provide data regarding the EIP. Preliminary data using decapitation, however, demonstrated a similar relationship to temperature as BTV and would be a more consistent assay for use in future experimentation with emerging *Culicoides*-borne pathogens.

3.1: Methods

Initially, trials were conducted using KC-*C. sonorensis* cell lines that had been used to screen the replication of BTV strains at constant temperatures as part of a previous Defra contract (SE: 2616). Eight flasks of KC-*C. sonorensis* cells were initially prepared and inoculated with an SBV strain (KC_1BHK_4: C_q = 14.81). The strain of SBV used was the same as that used in Objectives (1) and (2). Two 125cm^2 flasks containing 30ml of maintenance media and 400µl of inoculum were then incubated at each of four different constant temperatures (12, 15, 20 and 25°C). At two day intervals, supernatant was collected from each incubator and their RNA quantified using qPCR (as for Objective One) to a maximum of 22 days post-infection. This trial was then repeated using a ten-fold dilution of the same strain.

Infections were also conducted with *C. sonorensis*, which had been identified as a model species for work with SBV. Over one thousand *C. sonorensis* were initially fed through a membrane on blood/SBV mixtures using the same strain as in Objectives (1) and (2). These insects were divided roughly equally into groups of 50 individuals each to be stored at 15, 20 and 25°C. At specific times following introduction, batches of 25 potentially infected *C. sonorensis* were allowed to feed on FTA cards as trialled in Objectives (1) and (2) with detection being carried out via qPCR. Timing of testing was related to results already available from a previous project carried out with bluetongue virus (SE: 2616) on a comparative basis as follows: 15°C days 9, 13, 15, 17, 20, 21, 24, 26; 20°C days 4, 7, 8, 11, 13, 15; 25°C days 3, 6, 7, 8, 9, 10. In addition, at days 9 and 21 for 15°C, days 4 and 11 for 20°C and 3 and 7 for 25°C, 25 *Culicoides* were decapitated and the heads assayed for virus as in Objective (2).

3.2: Results

Despite having been used successfully for infection of *C. sonorensis* in objectives (1) and (2) there was no evidence of replication of SBV when applied to a cell line derived from the same species (Figures 3A and B). Following initial failure of infection in 3A, the strain was diluted in 3B in an attempt to detect whether replication was masked by a high initial inoculum and an additional preliminary experiment was conducted to assess whether replication was occurring within cells but was not detectable in the cell supernatant. Treatment with sonication did not result in a reduction in C_q values indicating that it was a lack of infection that caused the failures. While SBV had been found to replicate rapidly in the BHK-21 cell line maintained at The Pirbright Institute, this was not suitable for use in this trial due to the requirement for incubation at a range of temperatures. More recent studies have demonstrated that SBV will replicate to high levels of RNA on mosquito-derived lines such as C6-36 from *Aedes*. The lack of congruence between replication in vectors and derived cell lines indicates that an initial screening step of available cell lines would be useful when receiving novel viruses, particularly as it was suggested that isolation of SBV had originally been made on the cell line used in this study.

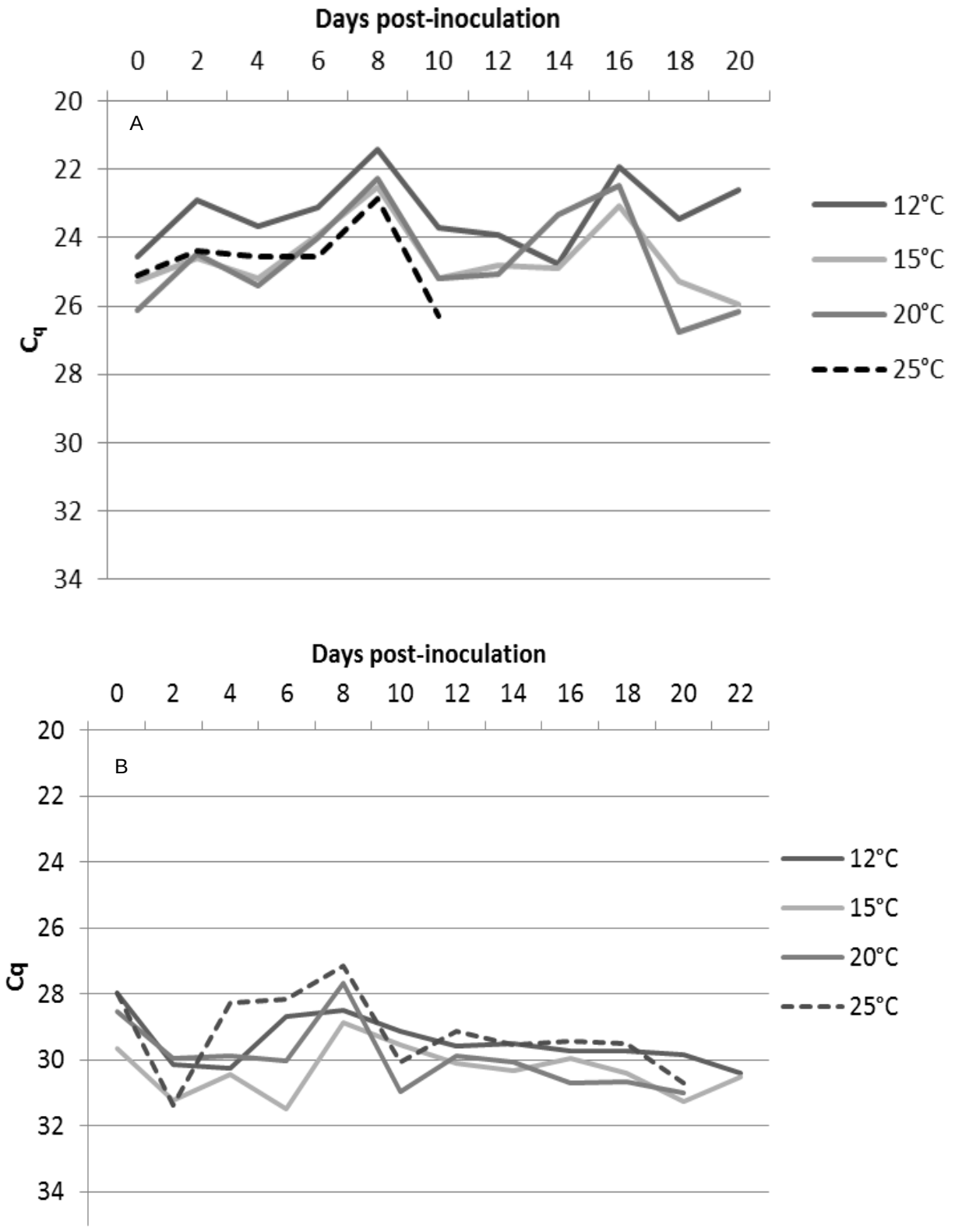


*Figure 4. Replication of an SBV isolate (A) and a ten-fold dilution (B) on KC-*C. sonorensis *cell line. No evidence of replication was detected at either concentration.*

FTA cards used for detection of completion of the EIP failed to produce convincingly positive results with no detection of SBV RNA from *C. sonorensis* incubated at 25°C and only trace levels (35.99->40) recovered from 15 and 25°C. When taken in context with the results of Objectives (1) and (2) it is clear that the sensitivity of this technique is extremely limited. In twenty-five *C. sonorensis* dissected after 7 days post-feeding at 25°C, eight individuals had evidence of infections in heads with C_q values ranging from 21.53-38.79. This did not result in detection via the FTA card and implies that utility of the cards will be reliant upon more than 8 fully disseminated individuals feeding. As this is extremely unlikely to occur in field collections and will require substantial numbers of laboratory individuals there is no clear role for this

technique without significant modification. Dissections of *C. sonorensis* incubated at other temperatures conformed with data previously generated for BTV (Table 1). Following an eclipse phase where SBV RNA from the original blood-meal was cleared from the *C. sonorensis* (evident in high C_q values recorded at day 9 at 15°C and day 3 at 25°C, fully infected individuals were detected at days 11 and 7 for 20 and 25°C, respectively.

Temperature (°C)	Day	Proportion containing SBV	C_q range
15	9	0.12	34.21-35.95
15	21	0	-
20	4	0	-
20	11	0.2	34.91-21.87
25	3	0.16	34.06-36.02
25	7	0.32	38.79-21.53

Table 1. SBV RNA in C. sonorensis *heads of individuals incubated at three temperatures following feeding on a blood/SBV mixture through a membrane.*

4. To conduct a preliminary investigation into transovarial transmission of SBV in putative vector species.

A preliminary investigation was made into the potential for transovarial transmission of SBV as an alternative means of overwintering in the virus. While no *Culicoides*-borne pathogen has been demonstrated to be capable of being transmitted transovarially, studies have almost entirely concentrated upon BTV as a target organism. In this study we processed several hundred eggs of *C. sonorensis* laid by membrane-fed individuals as a means of assessing the likelihood of this occurrence for SBV. No evidence was found of SBV RNA in processed eggs.

4.1: Methods

Approximately 100 female *C. sonorensis* were fed on a blood/SBV mixture using a Hemotek feeder as for Objectives (1), (2) and (3). These were then incubated for seven days at 25°C, allowing completion of the EIP, oogenesis and oviposition to occur. Approximately 400 eggs were collected from the pillbox containing the *C. sonorensis* (from a likely total of several thousand), divided roughly equally into two groups and homogenised by crushing between microscope slides and then in a TissueLyser® for one minute at 25Hz. Samples were then centrifuged and the supernatant tested for SBV RNA presence as for Objectives (1), (2) and (3).

4.2: Results

No evidence of SBV RNA was detected in eggs produced from adult *C. sonorensis*. While the number of *C. sonorensis* producing egg batches was not recorded, the vast majority of individuals tend to produce egg batches under the conditions described, although the proportion of fully infected individuals was not known. A key point in interpretation of the data lies in the processing of *Culicoides* eggs for examining evidence of infection which has not been standardised and presented substantial difficulties due to their small size. Future studies to be conducted under SE: 2618 with BTV should allow a more detailed investigation of this area and provide techniques that could be used to further investigate this area with SBV.

5. To produce a suitable high-throughput and sensitive assay for detection of SBV specific antibodies in animals previously infected by the virus.

Summary: This objective aimed to develop an assay for detection of SBV antibodies based on a competition/blocking ELISA format using recombinant expressed proteins and monoclonal antibodies. Using these reagents, which have no potential for contamination with the original virus, the resulting assay has no disease security restrictions concerning the movement and sharing of reagents or the assay. Consequently the assay can be rapidly shared with colleagues in other laboratories.

5.1: Methods

The genes encoding the N, Gn and Gc proteins of Schmallenberg virus (SBV) were cloned in a baculovirus system and recombinant viruses were confirmed by sequencing of the gene inserts. The protein expression is under the control of the strong polyhedrin promoter. Proteins were overexpressed in sf9 cells. A preliminary assay (a western blot format) was set to assess their ability to detect antibodies to SBV from a panel of sheep sera naturally infected with SBV. Both N and Gn proteins were found to highly antigenic and detected anti-SBV proteins. The recombinant proteins carry a 6xHis tag at both amino and carboxy termini

and can therefore bind an anti-Histag antibody.

We developed a preliminary ELISA assay whereby Streptavidine coated 96-well plates were used to bind biotinylated mouse anti-6His tag antibody. Baculovirus infected sf9 cell lysates were then added in the wells and the proteins were captured through their 6xHis tag, leaving the specific SBV protein sequence accessible to specific anti-SBV antibodies. The wells were washed thoroughly to remove unbound cellular protein lysates.

5.2: Results

This ELISA identified antibodies to SBV efficiently. In our experience such a configuration of the ELISA allows detection of not only IgG but could also be used to identify IgM antibodies. The commercially available SBV ELISA based on bacterially expressed N protein has a sensitivity of 97-98%, missing therefore 2-3% of SBV seropositive cases as opposed to seroneutralisation assays considered as the gold standard. By mixing the baculovirus expressed N and Gn proteins the sensitivity of our ELISA was found to 99.8%. We are currently further assessing our ELISA using larger panels of antisera from sheep and cattle naturally infected with SBV at a farm at the Royal Veterinary College (see publications).

General Conclusions

Objectives (1), (2), (4) and (5) were completed with only minor technical revision from the original work-plan. Objective (3) proved to be problematic due to the failure of SBV to replicate on the KC-*C. sonorensis* cell line combined with a lack of sensitivity in the FTA card system for detecting SBV in saliva elicited from adults. At present our determination of extrinsic incubation periods in *Culicoides* therefore remains restricted to laborious techniques (in this case decapitation and subsequent detection of SBV in the head). A key inclusion in working with novel emerging arboviruses in future scenarios where replication characteristics are not known, is an initial investigation of both suitable cell lines for use in assays and suitable means of storage that does not result in substantial loss of infectivity. These issues caused significant delays in experimentation during the initial month of the project. In addition, the inclusion of experiments that would enable the use of SBV in blood taken from vertebrate hosts would have been a useful comparison to competence rates achieved using artificial feeding methods used during experimentation in the current project. This latter area is likely to be a focus of further research in the immediate future.

Work generated from this project has allowed a framework to be created for interpretation of field collected data on *Culicoides* infection with SBV. This was important as our understanding of the role of qPCR in identification of vector species prior to this project was lacking. Prior to the completion of this project, several groups has published papers demonstrating the presence of SBV RNA in heads of common farm- associated northern Palaearctic species of *Culicoides* including the *C. obsoletus*/*C. scoticus* complex. Our data underpinned these results by demonstrating that they were indicative of fully disseminated infections. This in turn implies that from an entomological viewpoint SBV transmission will be possible at the vast majority of British farms where these species are ubiquitous. The utility of *C. sonorensis* as a model organism for SBV replication was also demonstrated in preliminary studies of the extrinsic incubation period and transovarial transmission. Further examination of these areas in Europe will strengthen epidemiological models produced for SBV transmission and elucidate differences with BTV. Finally, the diagnostic tool developed in Objective (5) will be of substantial benefit in field identification of SBV infection as it has several specific advantages over current technologies.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

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