Applied Veterinary Virology: The isolation and identification of viruses using laboratory animals

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ETHICS AND REGULATIONS

Animal experiments always have serious ethical and welfare implications. The issue of pain and distress in animals subjected to experimental infection concerns both the general public and researchers. The outcome of the experiment has to justify the adverse effects on the animal. A researcher performing such experiments must be prepared and able to explain and justify why a particular study should be conducted.

Livestock and companion animals are the natural host species in which certain viral diseases occur and are therefore the preferred model in which to conduct experimental infection studies. Infection studies using for example livestock are however expensive and time consuming and limited numbers of animals are therefore generally included. In addition, the complexity of the biological system of the whole animal gives unpredictable results and high variability, and often low repeatability. Inbred mice are easier and cheaper to work with. Extrapolation of findings in mice to livestock must however be done with care, due to differences in the biology between mice and large mammals.

Animal experiments are regulated by national and international laws and regulations. Most agencies responsible for setting standards for the care and use of experimental animals require investigators to consider the justification of the experiment and to implement the concept of the 3Rs (Reduction, Replacement and Refinement). The general principle of the 3Rs namely “Reduction, Replacement and Refinement” was developed many years ago and has become widely accepted as ethical principles (Balls et al., 1995). The 3Rs have been defined as “all procedures which can completely replace the need for animal experiments, reduce the numbers of animals required, or diminish the amount of pain or distress suffered by animals in meeting the essential needs of man and other animals” (Smythe, 1978). In general, the 3R measures can be implemented to improve the welfare of animals. The 3Rs also contribute to the quality of research findings through improved study design, reduced variability and increased statistical power. The reader may consult the NORECOPA (Norwegian Consensus Platform for Replacement, Reduction and Refinement of animal experiments) website (http://norecopa.no/about-norecopa) for detailed information on the rationale behind and implementation of the 3Rs. Special guidelines have further been developed to improve the study design, analysis and reporting of research using animals, the so-called ARRIVE guidelines (Killkenny et al., 2010). These guidelines are endorsed by an increasing number of scientific journals and may be consulted at the following URL: (http://www.nc3rs.org.uk/page.asp?id=1357).

A summary of the principles behind the 3Rs are provided below:

Replacement

Replacement refers to the replacement of animal experiments with non-animal alternatives, which can vary from computer models to less sentient animals or cell cultures. Ideally, all possible laboratory investigations should be performed before animal experiments are set up. Laboratory conditions are more controlled and the variability factors that complicate live animal research are reduced. However, *in vitro* testing can only partly provide a surrogate for *in vivo* infection.
Reduction

Reduction implies a decrease in the number of experimental animals without loss of information. This may be achieved through good experimental design and/or by controlling variation. The variation can be decreased by the use of genetically homogenous animals, by using controlled environmental conditions and/or by adhering to strict management procedures.

Refinement

Refinement refers to a change in scientific procedures and animal husbandry to minimize suffering, pain, stress and distress that the animals may experience. Refinement enables healthy animals with normal social behavior that also results in less variability with improved results. Relevant factors to consider will vary with the animal species and type of study. This could include appropriate use of anaesthetics, analgesics and other therapeutic measures, and the refinement of husbandry to improve the well-being of the animals (such as the use of environmental enrichment including litter and toys). Humane endpoints should further always be established to avoid unnecessary suffering for the animals.

In terms of the most basic requirements for the humane treatment of experimental animals, the following should be non-negotiable:

- Protect animals from cruel treatment
- Good food and clean water should always be available
- Protect animals from extreme weather conditions
- Pain must be minimized
- Animals must be treated with respect: you have no right to misuse them
- If tissue cultures can replace the experimental animal, it should be the preferred approach
- Animals in transportation require food and water

ANIMALS MOST COMMONLY SUBJECTED TO EXPERIMENTAL INFECTIONS OR LABORATORY TRIALS

Mice

BALB-c mice are white (albino) laboratory-bred strains of the common house mouse from which a number of common substrains are derived. Today it represents over 200 generations from its first description in New York in the 1920s. BALB/c mice are distributed globally, and are among the most widely used inbred mouse strains used in animal experimentation.
Rats

The rat is the second most cited animal model for experimental use. Compared to mice, rats are bigger, fiercer, and more resistant against various ailments. Sprague-Dawley rats and Wistar rats are the two most frequently used rat strains. Similar to the common mouse strains, these two rat strains are albino rats. On the other hand, both rat strains are outbred strains (most commonly used mouse strains are inbred strains). Laboratory rats are derived from the species Rattus norvegicus.

Guinea pigs

Biological experimentation on guinea pigs has been carried out since the 17th century and was most extensively implemented in research and diagnosis of infectious diseases but, has since been largely replaced by other rodents such as mice and rats. Guinea pig strains used in scientific research are primarily outbred strains. Aside from the common American or English stock, the two main outbred strains in laboratory use are the Hartley and Dunkin-Hartley; these English strains are albino, although pigmented strains are also available. They are still used in research, primarily as models for human medical conditions.

Rabbits

The rabbit (Oryctolagus cuniculus) is a standard laboratory animal in biomedical research and transgenic rabbits are used as animal models for a variety of human diseases both genetic and acquired. Classical experimental use of rabbits includes antibody production, development of new surgical techniques, physiology and toxicity studies for the testing of new drugs.

Each of these groups of animal has its own special husbandry needs.

EXPERIMENTAL WORK WITH VIRUSES

Isolation of viruses of veterinary importance is mostly done in sucking mice 1-3 days old. Examples of viruses that have been isolated in suckling mice include African horse sickness virus (AHSV), Rift Valley fever virus (RVFV) and Wesselsbron virus (WBV). For rabies virus, mice of 3-weeks-old are mostly used.

Antigen production is seldom done in laboratory rodents but when employed, 1-3-day-old suckling mice are used or baby mice 3 weeks old. Positive sera are traditionally produced in guinea pigs or rabbits via the intradermal, subcutaneous or intraperitoneal routes.

HANDLING OF SPECIMENS FOR VIRAL ISOLATION

It is important to register the specimen for later reference and know the history of the sample before handling it. It is advisable to work in a safety cabinet and to always use surgical gloves. These requirements simply ensure that samples are handled with care as one does not know what risks may be associated with the sample.
PREPARATION OF THE INOCULUM FOR VIRAL ISOLATION

Use a buffered medium such as buffered lactose peptone (BLP) to make a suspension of the sample (e.g. brain, spleen, liver, and lung). For general viral use, penicillin and streptomycin are added to suppress bacterial contamination. The recommended ratio is 100 ml BLP + 1 ml penicillin (5000 units) and 1ml streptomycin (5000 units). Then a 10% suspension of the organ is made (± 1 g organ + 9 g (9ml) BLP. Centrifuge at 2 000 rpm for 10 minutes. If the specimen is known or suspected to be contaminated, it can be filtered (0,22 µm sterile filter).

Blood may be used as is (undiluted), but if only white blood cells are required, the blood must be centrifuged, and the buffy coat removed and suspended in BLP.

ROUTES OF INOCULATION

There are seven routes of inoculation that may be used and include:

- Intracerebral (i/c)
- Intraperitonial (i/p)
- Intramuscular (i/m)
- Subcutaneous (s/c)
- Intradermal (i/d)
- Per os (p/o)
- Intravenous (i/v)(seldom used)

**Intracerebral**

The route is used frequently for virus isolation. Rats and mice are the preferred species and should be either 1-3 days old or 3 weeks old. The volume administered is 0,03 ml for mice and 0,05 ml for rats. The syringe is filled with the inoculum (needle 25-27G). The head is immobilized between the thumb and forefinger and the needle inserted into the area where the three cranial bones meet, only up to the depth of the needle bevel. After inoculation the mice or rats must be returned to its original cage.

**Intraperitoneal**

This is a route used normally for antibody production but can also be used for viral isolation. The species targeted for this purpose include female guinea pigs or rabbits and mice. The inoculum is combined with Freund’s adjuvant. The method for antibody production involves the simultaneous intraperitoneal inoculation of mice with a sarcoma cell line (sacoma 180) for the production of ascitic
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fluid. This leads to the development of a large volume of fluid with antibodies in the peritoneal cavity of the mouse. Mice of 6-8 weeks are used for i/p inoculation. However, due to animal welfare concerns, this method is today frowned upon.

For the intraperitoneal approach two persons are ideally involved. The first person will fill the syringe with the inoculum (± 3-10 ml). The second person will scruff the animal’s skin at the back of the neck with one hand and take the hind legs in the other. The animal is turned upside down so that the intestine is lower than the head. The abdomen is then presented to the first person who inserts the needle off-centre to the L/R of the midline (0,2 mm) to prevent damage to the intestines, urinary bladder or uterus. Never use rabbit ears as handles! When attempting to propagate RVFV or WBV, 3-week-old mice can be used and the liver can be subsequently harvested to demonstrate the virus.

**Intramuscular**

This approach is mainly used for antiserum production. Species used are mice, rats, guinea pigs, rabbits – preferably rabbit and guinea pig hind legs. Adult animals are preferred. One person must immobilize the animal, and the second person will aspirate the inoculum into the syringe and push the needle halfway into the muscle (the thickest part of the upper hind leg) and release the inoculum.

**Subcutaneous**

Mainly used for demonstrating antibodies in the serum after 21 days. The species used may include sheep or goats (free of antibodies against the virus to be tested) and adult animals are preferred. The first person places the sheep/goat in a sitting position, exposing the inner thigh. The second person disinfects the inoculation area with 70% alcohol. The skin is lifted and needle inserted between the skin and muscle. This method has been used for bluetongue virus (BTV), Rift Valley fever virus, epizootic haemorrhagic disease (EHD) virus and Wesselsbron virus. It is especially useful when large numbers of specimens are to be tested.

**Intradermal**

This route is used for antiserum production in adult rabbits or guinea pigs. The hair must be removed with hair remover (e.g. Nu-hair) and washed thoroughly and dried. On the second day the area must be disinfected with alcohol. The first person will hold the animal and the second person will have the inoculum ready in a syringe with a 25-27G needle the skin is pulled between the fingers and a little pressure applied until the skin is discoloured. Insert the needle between the layers of skin. Release the skin and push in the plunger. If a circumscribed discoloured area appears then it was correctly done, but if a lump appears then it was injected s/c. Viruses such as lumpy skin disease virus (LSDV) administered to rabbits or guinea pigs and foot and mouth disease virus (FMDV) in cattle tongue are inoculated /identified in this way.
ISOLATION OF VIRUSES OF VETERINARY IMPORTANCE

Laboratory animals can be used to isolate the following viruses of veterinary importance:

<table>
<thead>
<tr>
<th>Family</th>
<th>Flaviviridae</th>
<th>Coronaviridae</th>
<th>Rhabdoviridae</th>
<th>Paramyxoviridae</th>
<th>Bunyaviridae</th>
<th>Reoviridae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genus</td>
<td>Flavivirus</td>
<td>Coronavirus</td>
<td>Lyssavirus</td>
<td>Paramyxovirus</td>
<td>Phlebovirus</td>
<td>Orbivirus</td>
</tr>
<tr>
<td>Virus</td>
<td>Wesselsbron virus</td>
<td>Coronavirus</td>
<td>Rabies virus</td>
<td>New castle disease virus</td>
<td>RVFV</td>
<td>AHSV, BTV, equine encephalitis virus (EEV)</td>
</tr>
<tr>
<td>Lab. animal</td>
<td>Suckling mice</td>
<td>Suckling mice</td>
<td>Suckling mice</td>
<td>Chickens</td>
<td>Suckling mice</td>
<td>Suckling mice</td>
</tr>
<tr>
<td>Age</td>
<td>1 – 2 days</td>
<td>1 – 2 days</td>
<td>1 – 3 day old mice, or 3 week old mice</td>
<td>2 – 3 week old chickens, or embrionated chicken eggs</td>
<td>1 – 2 days</td>
<td>1-3 days old</td>
</tr>
<tr>
<td>Route</td>
<td>i/c</td>
<td>i/p</td>
<td>i/c</td>
<td>i/v / per os</td>
<td>i/c / i/p</td>
<td>i/c</td>
</tr>
</tbody>
</table>

Factors affecting the outcome of experimental infections

**Choice of animals:**

The most susceptible animal must be selected for a particular sample.

For example, 1 – 3 day old mice for RVFV and AHSV, 3 week old chickens for Newcastle disease virus or embrionated chicken eggs (9 days old) for BTV.

**Age of animals:**

The older the mice the less susceptible they become.

**Route of inoculation:**

The intracerebral route is the most common route for experimental infection. Dosage: 0.03 ml for suckling mice.
Time of sample collection:

The fresher the sample the more likely a virus will be isolated and the less likely bacterial contamination will take place.

**OBSERVATION OF INOCULATED ANIMALS**

Observe the animals daily after inoculation for the onset of clinical signs. If RVFV is suspected mice must be observed at least twice a day. Some viruses are slow growers in mice e.g. equine encephalosis virus (EEV) and can take up to 13 – 15 days to induce clinical signs. In some cases signs may only be present in 1 of a family of suckling mice. Other viruses grow fast and the onset of signs occurs within 36 hours e.g. RVFV, while AHSV may take 3 – 5 days.

**Evidence for successful propagation of the virus**

The first signs of infection are dependent on the nature of the virus, the route of inoculation and the dosage. The smaller the dosage the longer it takes for clinical signs to develop. Mice normally show nervous signs. The severity of the signs may differ and in many cases experience is required to identify such animals.

AHSV induces a very active motoring activity and scratching movement, whereas RVFV induces paralysis even if in general they look healthy and on occasion have milk in their stomachs.

**Precautions**

Special care must be taken when inoculating laboratory animals. Spread of the virus may occur during inoculation as a result of leakage from the inoculation site. The virus may also spread in excretions or aerosols, via the teeth and claws or as a result of cannibalism. During euthanazia and the opening of the carcass an aerosol may be created that can spread the virus. Extra care must therefore be taken during a necropsy. Be careful not to spill infected tissues and fluids. It is advisable to autoclave the carcass and incinerates it, especially if you do not know what virus you are dealing with.

**General clinical signs that may be observed in 1-3 days to 2-week-old mice and rats following intracerebral inoculation**

During the early stage they may walk on their toes, become irritable or sensitive when pressure is applied to the tail. They may react to sharp sounds and may exhibit nervous signs such as paralysis of the limb and body and spasms.

At the harvesting stage, suckling mice may lie on their sides and scratch their sides frantically. They will be dehydrated and very sensitive when pressure is applied to the tail. Close to death they will be lying still and have a blueish (cyanotic) colour. No milk will be visible in the stomach (white band in the abdominal area)
CLINICAL SIGNS INDUCED BY SPECIFIC VIRUSES

*Family Reoviridae*

*Genus: Orbivirus*

*African horsesickness virus*

Signs, depending on the dosage appear after 3 – 5 days post inoculation. The first sign is a high stepping gait (walking on their toes). They become sensitive to touch and sound and become dehydrated. In the terminal stages suckling mice are normally lying on their side and frantically move with a ‘scratching’ movement. They are dehydrated and very sensitive to touch. They will have no milk in their stomachs. Their colour also becomes cyanotic (normally pink).

*Genus: Reovirus*

*Reovirus of ruminants*

The virus may on occasion emerge in groups of mice. Baby mice appear normal. The only visible signs of infection are that they are irritable and their fur has a ‘wet look’. These signs appear after 10 –15 days post inoculation. Unfortunately this is not conclusive evidence especially when a colony of mice is contaminated with this virus. In this case the ‘wet look’ will appear with any stress within the colony.

*Family Flaviviridae*

*Genus: Flavivirus*

*Wesselsbron virus (WBV)*

Signs appear 36 hours to 3 days post inoculation and are similar to the signs observed with RVFV – see below

*Family Bunyaviridae*

*Genus: Phlebovirus*

*Rift Valley fever virus (RVFV)*

Signs appear 2 -5 days post inoculation. The signs of RVFV and WBV infection are basically the same. The onset of the clinical signs includes irritability, reactions to sound and flaccid paralysis of the limbs.

During the terminal stages flaccid paralysis of limbs and body may be observed. These mice are normally not dehydrated and can have milk in their stomachs. No movement is visible and within 2 – 3 hours after the onset of clinical signs, they will die.

*Family Coronaviridae*
**Genus: Coronavirus**

Signs can appear after 3 – 15 days. The signs caused by coronaviruses are similar to those described for reoviruses except the fur will turn yellow because of the diarrhoea. These signs are not very conclusive since laboratory mice colonies which are contaminated with the virus show clinical signs under stress. If the contamination is high the mice can die of anal blockage.

**Family Rhabdoviridae**

**Genus: Lyssavirus**

**Rabies virus**

Signs appear between 3 – 10 days depending on the dosage and age of the mice. Three-week-old mice as well as 1 – 3 day old suckling mice can be used. Signs such as aggressive behaviour and spasms followed by flaccid paralysis of the limbs are well recognized. Mice become dehydrated and the fur appears dull. There will be no milk present in their stomachs.

**Family Paramyxoviridae**

**Genus: Paramyxovirus**

**Newcastle disease virus**

Embryonated chicken eggs are normally used for isolation, but 2 – 4 weeks old chickens can also be used. Clinical signs start with coughing and later on flaccid paralysis of the limbs and spastic kicking of the legs occur.

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**HARVESTING OF ORGAN SAMPLES (SUCKLING MICE & RATS)**

**Brain: (Orbi- and flaviviruses)**

Following euthanization (where required) the animal is placed on a paper towel and disinfected with 70% alcohol. The skull is opened with sharp pointed scissors. Apply pressure on the skull with the forceps and scissors. If the brain is collected on the scissors release pressure on skull and deposit brain material in a marked container.

**Brain: (Rabies virus)**

Following euthanization and disinfection, the skull is opened and the brain removed without damage. The brain is cut into sections and placed on a paper towel. Impression smears are made on marked microscope slides for staining with fluorescent antibody conjugates.

**Liver: (e.g. RVFV)**

Following euthanasia, the animal is placed on its back and disinfected on a paper towel. The internal organs are exposed and the liver removed without damaging the gall bladder (rats do not have a gall
bladder). Close bile duct with forceps, remove and discard the gall bladder. Deposit the liver in a marked container. The liver may have a yellowish colour.

**Blood: (guinea pigs/rabbits generally harvested for serum antibodies)**

The animal is anaesthetized with CO₂/chloroform/ether and placed on its back. Palpate the thorax to detect the heart. Depending on the animal species, the needle varies from a 14G – 22G (1½ inch) - the size of the syringe will also vary. Insert the needle into the heart or in the case of large animals use the neck. When blood collects in the syringe, gently apply negative pressure.

Note: if whole blood is needed remember to use an anti-coagulant.

**CONFIRMATION OF THE PRESENCE OF THE VIRUS IN HARVESTED TISSUES**

A variety of confirmatory tests is available and will be determined by the specific virus one is trying to identify. The following are examples.

Electron microscopy: This method is normally used for the identification of enteric viruses such as parvo-, rota- and coronaviruses.

Haemagglutination and haemagglutination inhibition tests: This method is used in particular for members of the family *Paramyxoviridae* but is not exclusive for this family.

Complement fixation tests: The CFT is used less these days, but has in the past been used particularly for AHSV, RVFV and WBV.

Agar gel precipitation tests: These tests have likewise found application in some laboratories for AHSV, RVFV and WBV but are not used to the same extent anymore.

ELISAs: Viruses from the *Orbivirus* and *Flavivirus* genera have *inter alia* been identified with this method.