RAP publication 2002/22

# A BASIC LABORATORY MANUAL

# for

# THE SMALL-SCALE PRODUCTION AND TESTING OF I-2 NEWCASTLE DISEASE VACCINE

Sally E. Grimes


ISBN 974-7946-26-2

---

**For copy of the manual and correspondence, please contact:**

**Senior Animal Production and Health Officer**
**and Secretary of APHCA**
**FAO Regional Office for Asia and the Pacific (RAP)**
**39 Maliwan Mansion, Phra-Atit Road**
**Bangkok 10200, THAILAND**

**E-mail : aphca@fao.org**
**FAO Headquarters' Homepage : http://www.fao.org**
**FAO-RAP Homepage : http://www.fao.or.th**
**APHCA Homepage :** http://www.aphca.org

# A BASIC LABORATORY MANUAL

## for

## THE SMALL-SCALE PRODUCTION AND TESTING OF I-2 NEWCASTLE DISEASE VACCINE

# Table of contents

## Appendices

# List of figures

## List of figures (cont'd)

# Preface

The School of Veterinary Science at the University of Queensland has been involved in Newcastle disease virus research and the development of thermostable Newcastle disease vaccines for more than twenty years. The Australian Centre for International Agricultural Research (ACIAR) funded most of this research which was supervised by Peter Spradbrow and involved international collaboration. ACIAR supported the development of the I-2 Newcastle disease vaccine master seed stored at the School of Veterinary Science. The master seed is distributed to collaborating institutions for use in producing thermostable vaccines to control Newcastle disease in village chickens in developing countries.

This manual summarizes the basic laboratory procedures used to produce and test experimental I-2 thermostable Newcastle disease vaccine in the research and training programmes associated with the John Francis Virology Laboratory. The content of the manual is based on an earlier manual written by Peter Spradbrow, Zuhara Bensink and myself. The original manual was prepared for use in a practical laboratory workshop held at the Poultry Reference Laboratory at Onderstepoort in the Republic of South Africa in 1995. The workshop was funded by ACIAR and was the first of a series of workshops that aimed to transfer the technical skills required for the small scale production and testing of Newcastle disease vaccine. The original manual was revised in response to comments from trainees and colleagues and used in several subsequent workshops.

After the second laboratory workshop funded by the Food and Agriculture Organization (FAO) of the United Nations, Denis Hoffmann, Senior Animal Production and Health Officer of the FAO Regional Office for Asia and the Pacific suggested the manual be updated and rewritten for publication on the Animal Production and Health Commission for Asia and the Pacific (APHCA) website. The original content has been expanded and it is hoped the changed format will be easier to follow and translate. Comments are welcome.

Sally Grimes
John Francis Virology Laboratory
School of Veterinary Science
University of Queensland
St Lucia 4072
Australia
s.grimes@mailbox.uq.edu.au

**July 2002**

## Acknowledgements

This project has taken longer than anticipated and I thank Denis Hoffmann for his patience and for giving me the opportunity to write it in the first place.

Special thanks to Peter Spradbrow for ongoing support and generosity with time and knowledge.

Thanks also to the following people who have all contributed to the project and offered support:

Robyn Alders, Mary Young, Joanne Meers, Graham Burgess, Philemon Wambura, Joram Buza, Halifa Msami, Joseph Awuni, S.B. Chamling Rai, Karma Rinzin, Berhan Demeke Meshesha and Denise O'Rourke.

Assistance with the illustrations was provided by Sharka Marvilla of Sharka Designs and Naomi Mason of the University of Queensland.

Photographs were taken by the author and are from a collection kept at the John Francis Virology Laboratory.

## Abbreviations

| | |
|---|---|
| **ACD** | Acid citrate dextrose anticoagulant |
| **ACIAR** | Australian Centre for International Agricultural Research |
| **$EID_{50}$** | 50 percent embryo infectious dose |
| **$ELD_{50}$** | 50 percent embryo lethal dose |
| **DGV** | Dextrose gelatin veronal solution |
| **GMT** | Geometric mean titre |
| **HA** | Haemagglutination / haemagglutinin |
| **HI** | Haemagglutination inhibition |
| **IU** | International units |
| **$LD_{50}$** | 50 percent lethal dose |
| **MDT** | Mean death time |
| **OIE** | Office international des epizooties |
| **PBS** | Phosphate buffered saline |
| **PCV** | Packed cell volume |
| **PSG** | Penicillin Streptomycin Gentamycin antibiotic solution |
| **RBC** | Red blood cells |
| **SPF** | Specific pathogen free |
| **V4-HR** | Heat resistant strain of V4 Newcastle disease virus vaccine |
| **+ ve** | Positive |
| **- ve** | Negative |

# 1. Newcastle disease vaccines: an overview

## Introduction

Chickens are susceptible to many infectious diseases. One of the most important of these is the viral disease known as Newcastle disease, which causes devastating losses in both commercial and village chickens. Reducing losses of large numbers of village chickens to virulent Newcastle disease is an essential first step to improving their productivity. Newcastle disease can be controlled by the use of vaccines. There are many Newcastle disease vaccines suitable for use in commercial chickens. These are available on the international market. The I-2 Newcastle disease vaccine has been developed for local or regional production and use in controlling Newcastle disease in village chickens.

Many Newcastle disease vaccines deteriorate after storage for one or two hours at room temperature. This makes them unsuitable for use in villages where the vaccine may need to be transported for hours or in some cases days at ambient temperature. The I-2 Newcastle disease vaccine is more robust and is known as a thermostable vaccine. Thermostable vaccines still require long-term storage in the refrigerator. However during transportation of the vaccine to the field, the vaccine will not deteriorate as quickly as the traditional vaccines. Evaporative cooling provided by wrapping the vaccine in a damp cloth will be adequate for maintaining the viability of the vaccine during transportation to remote villages. However if it is stored in direct sunlight or allowed to reach high temperatures (above 37$^{o}$C) for more than a few hours it too will deteriorate and be unsuitable for use as a vaccine.

## Immunity to Newcastle disease virus

Chickens that survive infection with virulent Newcastle disease virus develop a long lasting immunity to further infection with Newcastle disease virus.

The basis of this immunity is:

1. Circulating antibodies.
2. Secreted antibody producing mucosal immunity.
3. Cell mediated immunity.

Newcastle disease virus of low virulence induces similar immune responses without causing severe disease. This is the basis of vaccination.

See **Appendix 2** for more information about Newcastle disease virus.

## Live vaccines

These vaccines are made with virus that is alive and able to infect cells. Strains of virus of low or moderate virulence are used. They mimic natural infection and induce all three immune responses.

## Killed vaccines

The ability of the virus to infect cells has been destroyed by treatment with a chemical, radiation or heat. These vaccines invoke only a circulating antibody response.

## Some vaccine strains of Newcastle disease virus

Strains of Newcastle disease virus have been broadly classified into four pathotypes as follows:

Avirulent Causes no disease

Lentogenic Low virulence, low mortalities, loss of egg production

Mesogenic Moderate virulence, mortalities up to 50 percent, loss of egg production

Velogenic High virulence, severe disease with high mortalities.

(Spradbrow P.B. 1987)

Many strains of Newcastle disease virus other than velogenic strains are used in the production of live vaccines. Eight of these strains are listed in **Table 1**.

## Table 1: Eight strains of Newcastle disease virus used in live vaccines

| Strain | Description |
|---|---|
| F | Lentogenic. Usually used in young chickens but suitable for use as a vaccine in chickens of all ages. |
| B1 | Lentogenic. Slightly more virulent than F, used as a vaccine in chickens of all ages. |
| La Sota | Lentogenic. Often causes post vaccination respiratory signs, used as a booster vaccine in flocks vaccinated with F or B1. |
| V4 | Avirulent. Used in chickens of all ages. |
| V4-HR | Avirulent. Heat Resistant V4, thermostable, used in chickens of all ages. |
| I-2 | Avirulent. Thermostable, used in chickens of all ages. |
| Mukteswar | Mesogenic. An invasive strain, used as a booster vaccine. Can cause adverse reactions (respiratory distress, loss of weight or drop in egg production and even death) if used in partially immune chickens. Usually administered by injection. |
| Komarov | Mesogenic. Less pathogenic than Mukteswar, used as booster vaccine. Usually administered by injection. |

## Thermostable Newcastle disease vaccines

Thermostable Newcastle disease vaccines exhibit a relative resistance to inactivation on exposure to elevated temperatures. Strains of Newcastle disease virus vary in thermostability.

- Thermostable vaccines are prepared from a strain of Newcastle disease virus that retains its ability to infect cells after storage outside a cold chain for a short period of time.
- There are two basic processes used to produce a thermostable Newcastle disease vaccine.
    1. Isolation of naturally occurring thermostable variants of the virus.
    2. Increasing the thermostability of this variant by artificial selection in the laboratory.

- A seed lot system is used to produce the vaccine. The sequential use of a master seed and a working seed minimizes the number of passages to produce a vaccine and maintains the genetic stability of the vaccine virus. The antigenicity and the thermostability of the virus in the master seed should be retained during the two passages that produce the working seed and then the vaccine.

See **Section 13** for more details.

# 2. Basic laboratory skills

## Introduction

Laboratory staff should be familiar with and have practiced the following skills prior to the commencement of Newcastle disease vaccine production. This manual does not contain further details about these skills.

- Aseptic technique.
- Sterilization by autoclaving and hot air of glassware and discarded materials.
- Mixing or stirring by:
  1. Hand
  2. Magnetic stirrer
  3. Vortex mixer
- Measuring pH
- Using centrifuges

## Instructions for using a daybook

The details of all technical procedures carried out to produce and test the I-2 Newcastle disease vaccine must be recorded by the technician in a daybook.

- Use a bound book.
- Sequentially number all the pages.
- Set aside 3 to 4 pages for a “table of contents”.
- Set up an “abbreviations page” at the front of the daybook. List all personalized or uncommon abbreviations.
- Put the date and title of the experiment at the top of each page.
- It is best to use a black pen as this colour photocopies well. Do not use pencil.
- Errors should be ruled through and corrected. Do not use an eraser or cover mistakes with white “liquid paper”.

## Preparation of reagents

The following reagents will be required during the production and testing of the I-2 Newcastle disease vaccine.

- Phosphate Buffered Saline (PBS)
- An anticoagulant. Acid Citrate Dextrose (ACD) or alternatively Alsever's Solution
- Dextrose Gelatin Veronal (DGV). A storage solution for 10 percent washed red blood cells
- An antibiotic solution
- Tryptic soy broth
- Sabouraud agar

Methods for preparing these media and solutions are included in **Appendix 3.**

## Recording details of practical work

Use the following format for recording the details of your practical work.

**Aim:** Outline the purpose of the activity.

**Materials and Methods:** Record enough information that will allow another scientific or technical person to repeat the procedure. If standard procedures are used in a series of experiments, describe them as standard. Examples of such standard procedures are those described in a Laboratory Manual or a previous experiment.

**Results:** Record all the results and observations. Prepare a table for recording data where appropriate.

**Conclusions:** Comment on your results and observations. Make suggestions for further experiments.

## The care and use of single and multichannel micropipettes

Manufacturer's instructions are supplied with all micropipettes. Please read and make sure you understand how to operate and care for the pipette. Micropipettes use plastic disposable tips. For ease of use, tips are usually packed into plastic boxes that can be autoclaved. Make sure the tips you are using will fit tightly onto the end of the pipette.

- Treat micropipettes very gently as they are precision instruments.
- Keep upright when in use to prevent liquids running inside the shaft of the pipette.
- Do not leave pipettes lying on the workbench where they can be knocked off and damaged.
- Do not allow pipettes to come into contact with corrosive chemicals.
- Before use, make sure the volume has been correctly set. Adjust the volume before use. Most pipettes have a digital display of volume. Some brands have a micrometer setting, which can be difficult to read.
- Check all tips are securely fitted to pipette.
- Draw liquid up.
- Check that the liquid drawn up has reached the expected level in the tip and there are no air bubbles in the tip. When using multichannel pipettes, check that the volume of liquid is the same in each tip.
- If necessary, expel the liquid and manually tighten the tips onto the pipette.
- Draw up the liquid and check again.

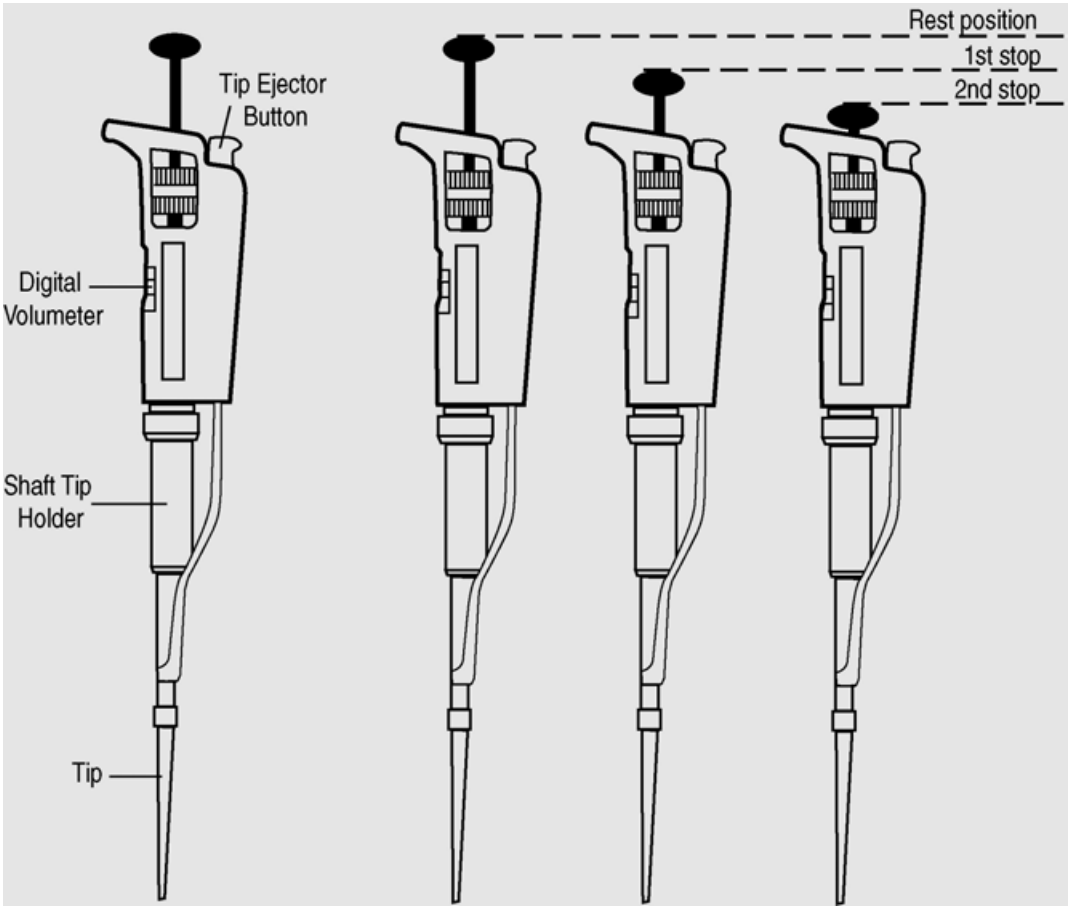
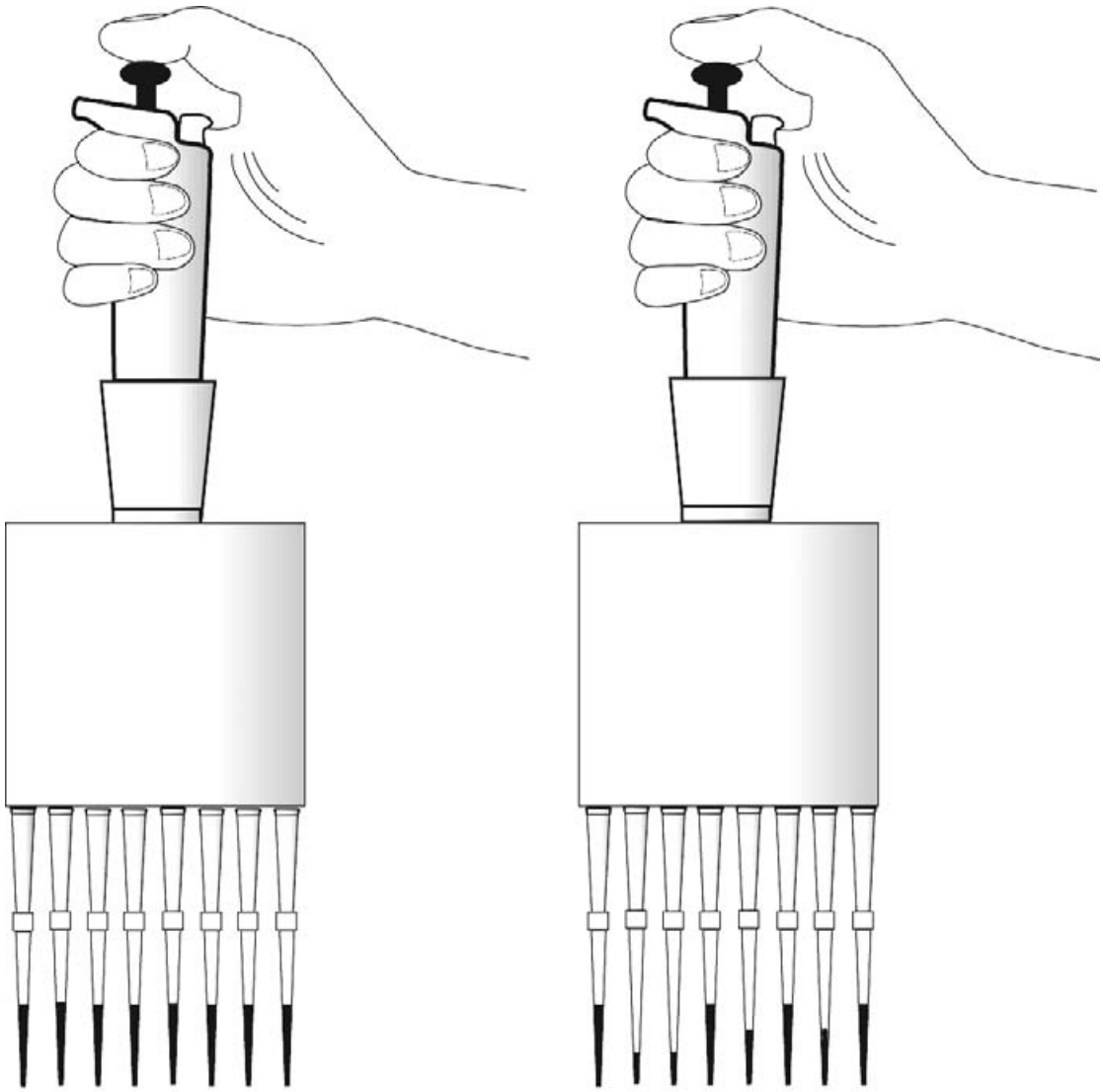



**Figure 1: Single channel micropipette**

## Instructions for pipetting liquids using a micropipette

1. Micropipettes have 3 positions:
    1. Rest position
    2. First stop
    3. Second stop
2. Fit the tip to the end of the shaft. Press down and twist slightly to ensure an airtight seal.
3. Hold the pipette in a vertical position. Depress the plunger to the first stop. Air equal to the volume of the setting (e.g. 100 μL) is displaced.
4. Immerse the tip into the liquid. Release the plunger back to the rest position. Wait a second for liquid to be sucked up into the tip. The volume of liquid in the tip will equal the volume of the setting of the micropipette.
5. Place the tip at an angle ($10^{o}$ to $45^{o}$) against the wall of the vessel receiving the liquid, for example a well of a microwell plate. Depress the plunger to the first stop, wait one second, press the plunger to the second stop to expel all the liquid
6. Move the end of the tip away from the liquid. Release the plunger to the rest position.




Each tip has drawn up an equal volume of liquid.

The volume of liquid drawn up in each tip varies.

**Figure 2: Using a multichannel micropipette**

## Graduated pipettes

Graduated pipettes are calibrated and marked with graduation lines that allow the measurement of more than one volume. The volume is read by eye by reading the value indicated on the scale at the bottom of the meniscus. Disposable plastic graduated pipettes are available and are useful for pipetting toxic or viscous substances. Graduated pipettes made of glass can be washed and reused. The pipettes should be plugged with cotton wool on the top and sterilized before use to minimize contamination of fluids being measured.

1 mL and 10 mL graduated pipettes are most commonly used in the laboratory practices described in this manual. Before using graduated pipettes check the volume scale and note:

- Does the pipette empty from full volume to zero or from zero to full volume?
- Is the pipette designed to be emptied by gravity with the tip in contact with the vessel or to be expelled by blowing out with a pipette filler?

## Pasteur pipettes

Pasteur pipettes are glass pipettes used to transfer fluids from one place to another. They are not graduated and are therefore not used to measure volumes. Like graduated pipettes they should be plugged with cotton wool and sterilized before use.

## Pipette fillers

Pipetting by mouth is not an acceptable laboratory practice. Fluids are drawn up into pipettes using pipette fillers. There are several options. A simple rubber bulb is suitable for a 1 mL pipette. For 10 mL pipettes, use a triple valve rubber bulb, a hand operated pump or an electronic pipette filler.

## Microwell plates

Plastic microwell plates are now routinely used in laboratories to perform assays using small volumes of samples and reagents. The plates contain 96 wells arranged in an 8 x 12 format with columns labeled 1 to 12 and rows labeled A to H. Thus each well has a designation for example A 12. Plates are manufactured with different shaped wells suitable for different tests. The wells can be round bottom, flat bottom and V-bottom.

In this manual, the haemagglutination and haemagglutination inhibition tests are carried out in V-bottom microwell plates. Two-fold dilutions can be made across the plate in either orientation that is from A1 to A11, (A12 being a control well) or from A1 to G1 (H1 being a control well).

The microwell plates used at the John Francis Virology laboratory are manufactured by Nalgen Nunc International. The plates cannot be autoclaved and are disinfected after use by soaking overnight in a 2 percent chloride solution. They are then washed and rinsed three times in tap water, followed by rinsing three times in distilled water, dried and reused.

Use of microwell plates is referred to in **Section 10, Section 11 and Appendix 4.**

(For details about the cost and supply of microwell plates visit the Nalgen Nunc website at http://nuncbrand.com and look up the product catalogue number 442587.)

# 3 Laboratory safety: an overview

## Introduction

The person supervising the production and testing of the I-2 thermostable Newcastle disease vaccine is responsible for ensuring that laboratory staff are familiar with the laboratory safety rules and regulations. It then becomes the responsibility of each person involved with the production of the vaccine to work within this framework. Laboratory staff must give due consideration to their own and their colleague's safety. The maintenance of clean and tidy work areas is an important precaution that is easy to implement.

## Safety manual

Every laboratory should have a safety manual. Ensure all the procedures involved with the production and testing of I-2 Newcastle disease vaccine are covered in the laboratory safety manual.

**This manual does not take the place of a laboratory safety manual**

## A list of some of the safety issues to be addressed in a laboratory safety manual

- Fire
- First aid
- Safe use of laboratory glassware
- Safety with handling boiling liquids
- Cleaning up spills and broken glass
- Decontamination of benches and waste
- Recycling of infected materials for example, needles and syringes
- Safe use of chemicals

## A list of basic laboratory rules

- Always wash hands thoroughly with soap on entering and leaving the laboratory.
- Do not eat, drink or smoke in the laboratory.
- Always wear a laboratory coat.
- Label all reagents clearly with contents, date and the initials of the person who prepared the reagent.
- Do not pipette by mouth.
- Dispose of discarded materials as instructed.

## Human infection with Newcastle disease virus

There have been instances recorded of human infection with Newcastle disease virus. On one occasion, allantoic fluid containing virulent Newcastle disease virus splashed into the eye. A mild conjunctivitis resulted. The author is unaware of more serious infections with Newcastle disease virus. It is important to take care when handling live Newcastle disease virus including the I-2 strain. Avoid contact with the virus.

## Websites

More information about basic laboratory safety can be found at the following websites.

- University of Calgary

http://www.ucalgary.ca/~ucsafety/bulletins/genlab1.htm

- University of California

http://envhort.ucdavis.edu/safety/labsafety.html

- University of Sydney

http://www.usyd.edu.au/su/ohs/labsafety.html

- Centers for Disease Control and Prevention, Atlanta, Georgia, USA

http://www.cdc.gov/od/ohs/manual/labsfty.htm

- Howard Hughes Medical Institute

The Office of Safety at the Howard Hughes Medical Institute has a useful website with access to Laboratory Chemical Safety Summaries and training materials.

http://www.hhmi.org/research/labsafe/index.html

# 4 Incubation of fertile eggs

## Introduction

All strains of Newcastle disease virus grow in embryonated eggs. Embryonated eggs are used for isolating the virus, producing vaccine and producing antigen for serological tests.

## Source of eggs

There is a theoretical risk of contaminating vaccines with pathogens that are transmitted through embryonated eggs. The use of embryos from a Specific Pathogen Free (SPF) flock minimizes this risk. However, in many countries, embryonated eggs from an SPF flock are unavailable. In this case it is practical to use embryonated eggs from a healthy local flock.

When deciding which flock to use to supply the eggs, consider the following points. Seek veterinary advice about the health status of the flock.

- Does the flock appear healthy and free from infectious disease?
- Is there any serological testing performed on the flock?
- What is the vaccination regime used in the flock?
- Is the flock free of *Salmonella pullorum*?
- Are the eggs clean?
- What is the percentage of fertile eggs?
- Is the hatching rate acceptable?
- Are the newly hatched chicks healthy?
- Do not purchase eggs during an outbreak of any disease in the flock supplying the eggs.
- If eggs are purchased from a commercial hatchery at 8 or 9 days old, candle at the hatchery to select eggs with viable embryos. Keep the eggs warm during transport to the vaccine production centre.
- Eggs with white shells are preferable as they are easier to candle.

## Recording details of egg purchases

Once a suitable source of embryonated eggs has been located, an order can be placed for the delivery of the eggs. It is useful if the person responsible for placing the orders and receiving the eggs keeps records.

The following information should be recorded in a notebook set aside for this purpose.

- Date when the eggs are ordered and the name of the person who received the order.
- Number and age of the eggs ordered.
- Date and number of the eggs received.
- Colour and appearance of the eggs received.
- Number of eggs damaged during transport.
- Date and number of eggs placed in incubator.
- Number of viable eggs after candling prior to inoculation.

## Storage and cleaning of eggs

- Do not buy dirty eggs.
- Eggs that are stained can be disinfected by washing in a warm ($37^{o}C$) solution of 0.1 percent Chloramin B (benzine sulfonamide sodium salt) or wiped with a 70 percent alcohol solution.
- Fertile eggs that have not been incubated can be purchased. They can then be placed in an incubator when they are delivered. Alternatively, they can be stored for several days in cool conditions ($16^{o}C$ to $18^{o}C$) prior to incubation. This may reduce the number of viable embryos, as some embryos may not develop after storage.

## Incubation of eggs before inoculation

Many vaccine production centres will already have large commercial incubators installed. Smaller incubators are available and are suitable for the small-scale production of vaccine.

- Incubation temperature = 38$^{o}$C to 39$^{o}$C.
- Humidity should be maintained at 60 to 65 percent. A tray filled with water and placed in the bottom of the incubator is usually sufficient to maintain this level of humidity.
- Place the eggs in the incubator with the air sac on top.
- Eggs should be turned three times a day.

## Incubation of eggs after inoculation

Inoculated eggs contain virus and should be placed in a different incubator. Eggs inoculated with virulent strains of Newcastle disease virus should not be incubated in the same incubator as used for eggs inoculated with the avirulent I-2 strain of Newcastle disease virus.

Inoculated eggs are incubated under the same conditions as uninoculated eggs but do **NOT** turn the eggs.

## Cleaning and decontamination of incubators

Keep surfaces clean by wiping out with a wet cloth and disinfecting with 70 percent alcohol solution or a non-corrosive disinfectant.

See **Section 16** for more information about cleaning and decontamination.

# 5 Candling eggs

## Introduction

Candling is the process of holding a strong light above or below the egg to observe the embryo. A candling lamp consists of a strong electric bulb covered by a plastic or aluminium container that has a handle and an aperture. The egg is placed against this aperture and illuminated by the light. If you do not have a candling lamp, improvise. Try using a torch.

Candling is done in a darkened room or in an area shielded by curtains. **Figure 6** shows the candling booth at the John Francis Virology Laboratory.

## Determining the viability of the embryo

Under the candling lamp, the embryo appears as a dark shadow with the head as a dark spot. Healthy embryos will respond to the light by moving. Sometimes the movement is very sluggish and it can take 30 to 40 seconds for the embryo to move when held under the candling lamp. This indicates the embryo is not healthy and the egg should be discarded.

Look carefully at the blood vessels. They are well defined in a healthy embryo. After an embryo has died, the blood vessels start to break down. They then appear as streaks under the shell when viewed under the candling lamp. Candling will also reveal cracks in the eggshells. Eggs with cracked shells should be discarded.

- **Infertile eggs:** These are easy to detect, as the egg is clear. Discard
- **Early deaths:** The embryo has developed for several days and then died. Candling will reveal a small dark area and disrupted blood vessels. Often deteriorating blood vessels will appear as a dark ring around the egg. Discard.
- **Late Deaths:** These are often difficult to tell apart from a viable embryo at the same stage of development. Look for the absence of movement and the breakdown of the blood vessels. Discard
- **Viable Embryos:** These move in response to the light and have well defined blood vessels. Mark the air sac and the inoculation site and then return the eggs to the incubator ready for inoculation.

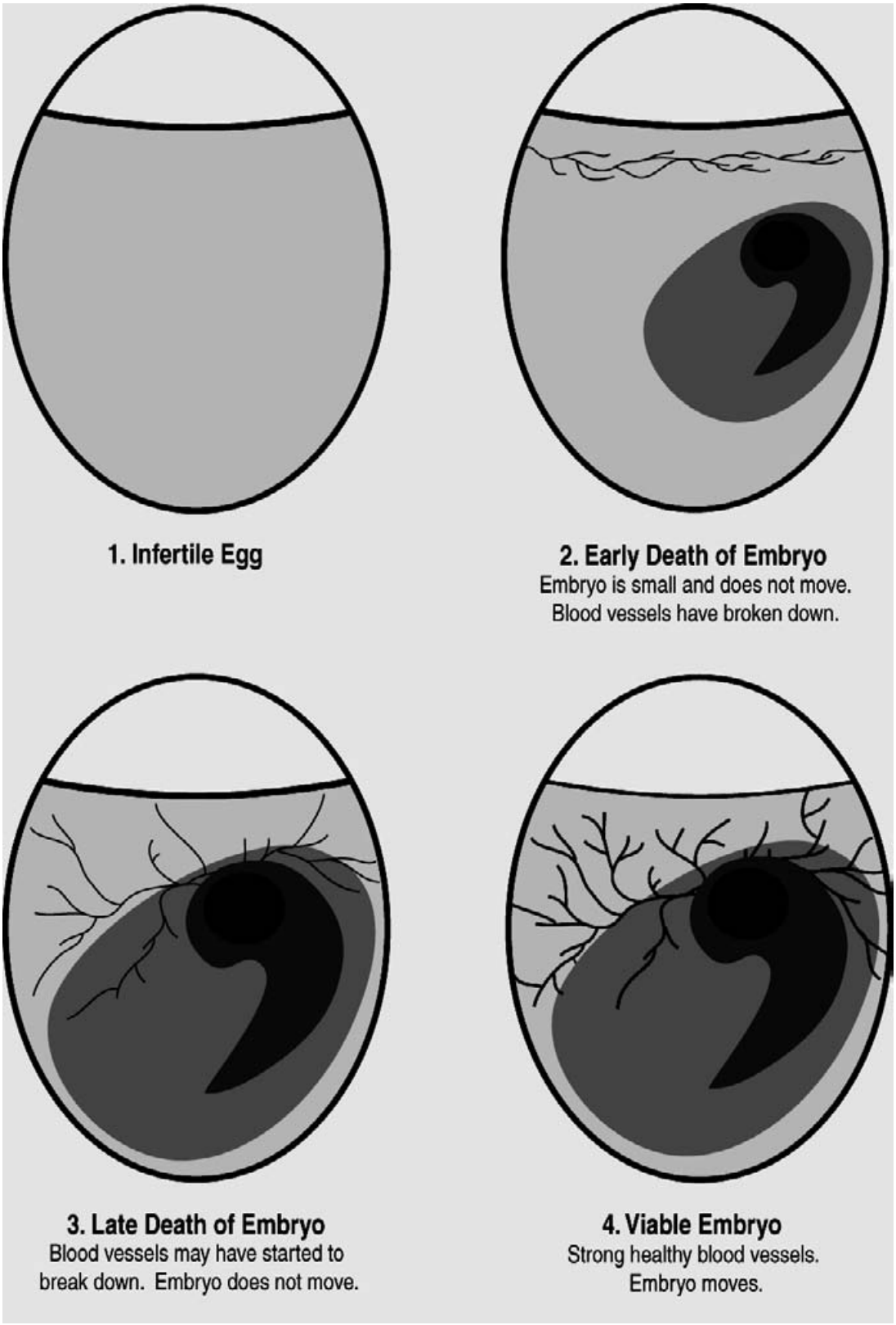



**Figure 3:**

**Features of embryonated eggs visible during candling**

## Marking the inoculation site:

1. Hold the blunt end of the egg against the aperture of the candling lamp and note the position of the head of the embryo.
2. Turn the egg a quarter turn away from the head.
3. Draw a line on the shell marking the edge of the air sac.
4. Draw an X approximately 2 mm above this line.
5. The X marks the inoculation site.

## Note:

- In some eggs the air sac will have not developed on the blunt end but half way down the egg. These eggs are not suitable for vaccine production. They can be used for inoculation during routine titrations to establish infectivity titres.

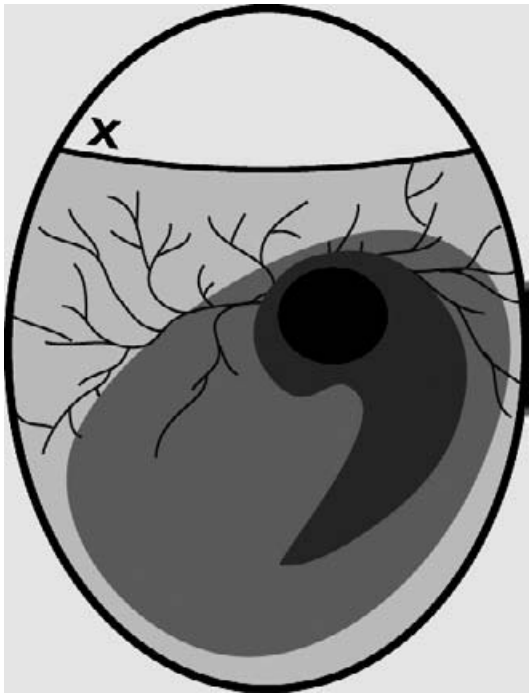



**Figure 4: Marking the inoculation site**

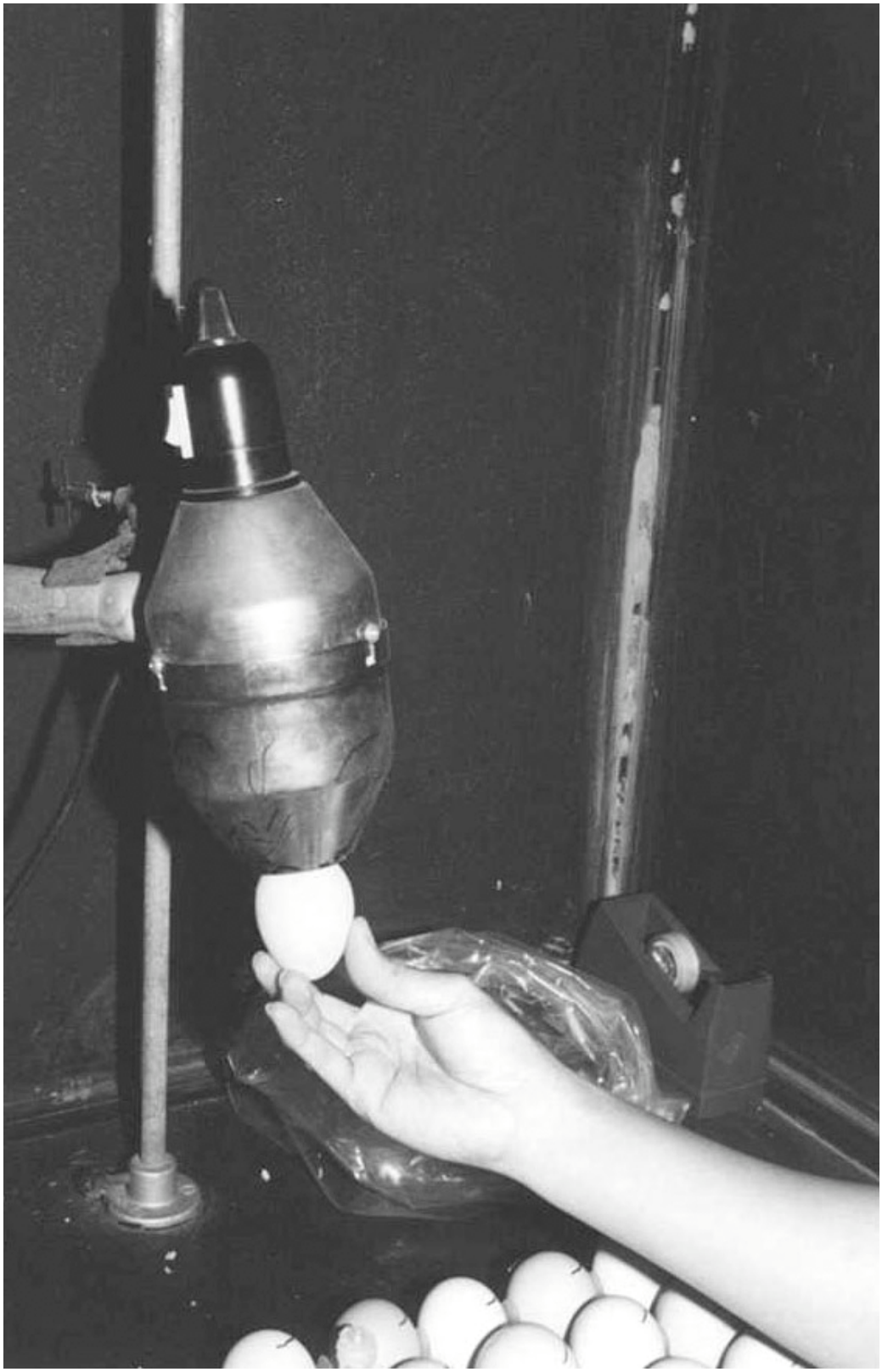


**Figure 5: Candling an egg**

**Figure 6: A candling booth**

# 6 Inoculation of embryonated eggs by the allantoic cavity route

## Introduction

The most convenient method of propagating Newcastle disease virus in the laboratory is by the inoculation of the allantoic cavity of embryonated eggs.

All strains of Newcastle disease virus will grow in the cells lining the allantoic cavity. The virus enters these cells where it multiplies. As the cells are disrupted the virus is shed into the allantoic fluid.

Virulent strains of the virus will invade cells beyond the lining of the allantoic cavity and kill the embryo. The time taken for this to occur is the basis of the "Mean Death Time Assays", which indicate the level of virulence.

The avirulent I-2 strain of Newcastle disease virus will not kill embryos inoculated by the allantoic cavity.

Inoculation of the allantoic cavity of embryonated eggs is a technique used in the following procedures:

1. Newcastle disease vaccine production
2. Establishing the infectivity titre of a suspension of Newcastle disease virus.
3. Isolation of Newcastle disease virus from field specimens for laboratory diagnosis.