Australian Government
Australian Centre for
International Agricultural Research

CONTROLLING NEWCASTLE DISEASE in Village Chickens

A Laboratory Manual

The Australian Centre for International Agricultural Research (ACIAR) was established in June 1982 by an Act of the Australian Parliament. ACIAR operates as part of Australia's international development cooperation program, with a mission to achieve more productive and sustainable agricultural systems, for the benefit of developing countries and Australia. It commissions collaborative research between Australian and developing-country researchers in areas where Australia has special research competence. It also administers Australia's contribution to the International Agricultural Research Centres.

Where trade names are used this constitutes neither endorsement of nor discrimination against any product by the Centre.

ACIAR MONOGRAPH SERIES

This series contains the results of original research supported by ACIAR, or material deemed relevant to ACIAR's research and development objectives. The series is distributed internationally, with an emphasis on developing countries.

First published in 2002. Second edition 2012.

Young M., Alders R., Grimes S., Spradbrow P., Dias P., da Silva A. and Lobo Q. 2012. Controlling Newcastle disease in village chickens: a laboratory manual. 2nd edn. ACIAR Monograph No. 87. Australian Centre for International Agricultural Research: Canberra. 143 pp.

ACIAR Monograph No. 87 2nd edition

ACIAR Monographs – ISSN 1031-8194 (print), ISSN 1447-090X (online)

ISBN 978 1 921962 43 1 (print)
ISBN 978 1 921962 44 8 (online)

Editorial production process managed by Melanie Lawrence.
Professional typesetting done by Beverley McIntyre, Wellington.

Foreword

Newcastle disease (ND) is a major constraint to poultry production worldwide. It is endemic in many developing countries and is of particular significance in villages where the livelihoods of people depend on poultry farming. Successful and sustainable control of ND in village chickens is achieved through vaccination and depends on a reliable, readily available and affordable supply of vaccine suited to the needs of village chicken owners. Local production of I-2 ND vaccine and its use in ND control programs are ongoing in a number of countries and have resulted in improved chicken production and improvements in the living conditions of many vulnerable rural families in developing countries. Such families benefit from enhanced nutrition and income generation through the sale of surplus chickens or eggs.

Over the last 15 years Australia has supported effective ND control programs in Asia and Africa including a number of research projects funded by the Australian Centre for International Agricultural Research (ACIAR). Research funded by ACIAR contributes to food and nutrition security, food safety and ecologically sustainable livestock production. This manual and its companion volumes contribute significantly to this work through enhancing local capacity to control ND and improve village chicken production leading to better nutrition, improved incomes and environmental conditions for those living in poverty.

Since its first publication in 2002, the manual has been used successfully in many African and Asian countries that both produce and use I-2 ND vaccine. The manual is recognised as a valuable source of information for laboratory personnel and has formed the basis of practical training workshops for scientists and technicians in vaccine-producing laboratories in Africa and Asia. This increased capacity allows countries to control the quality of both locally produced and imported vaccines, and ensure effective vaccine delivery in areas where cold chains are unreliable.

This second edition, updated on the basis of practical experience over the last decade, has been published due to increasing interest in local production of ND vaccine, particularly I-2. The French translation will also ensure that this information is readily accessible to francophone countries in the region.

Nick Austin
Chief Executive Officer
ACIAR

Acknowledgments

During the preparation of this manual, the authors drew on information contained in the manuals *Small-scale production and testing of Newcastle disease vaccine: Laboratory manual*, prepared by Peter Spradbrow, Zuhara Bensink and Sally Grimes (1995) and *Training course in local production of I-2 Newcastle disease vaccine* by Murray Maclean and Malcolm Ramsay (1999). Section 4.9 was contributed by Dr Ian Morgan and Section 4.8.3 by Dr Zuhara Bensink. We gratefully acknowledge their assistance. Special thanks go to Mr Razac Chame for his patience and artistic excellence.

We also wish to acknowledge the support, guidance and assistance of all who have been involved in our work:

- the Director of the Directorate of Animal Sciences in Mozambique
- staff of the School of Veterinary Science at the University of Queensland, in particular Dr Joanne Meers, and
- the Australian Centre for International Agricultural Research (ACIAR).

Table of Contents

Abbreviations

ACD	Acid-citrate-dextrose
ACIAR	Australian Centre for International Agricultural Research
AR	Analytical reagent
ASEAN	Association of South-East Asian Nations
°C	Degrees Celsius
DGV	Dextrose-gelatin-veronal
EID_{50}	Mean (50%) embryo-infectious dose
ELD_{50}	Mean (50%) embryo-lethal dose
°F	Degrees Fahrenheit
g	Grams
g	Acceleration due to gravity
GMP	Good manufacturing practice
GMT	Geometric mean titre
HA	Haemagglutination or haemagglutination test
HI	Haemagglutination inhibition
HN	Haemagglutinin neuraminidase glycoprotein on envelope of Newcastle disease virus
I-2	Thermotolerant, avirulent strain of Newcastle disease virus, used as a live vaccine
ICPI	Intracerebral pathogenicity index
IVPI	Intravenous pathogenicity index
L	Litre
Log	Logarithm
LDPE	Low density polyethylene
MDT	Mean death time
m	Metre
µL	Microlitre
mL	Millilitre
mm	Millimetre
Mol. wt	Molecular weight
MSDS	Material safety data sheet
ND	Newcastle disease
NGO	Nongovernmental organisation
NDV4-HR	Live, avirulent vaccine against Newcastle disease selected for enhanced heat resistance
OIE	World Organisation for Animal Health
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
pH	Measure of acidity or alkalinity of a solution
QA	Quality assurance
QC	Quality control
RBC	Red blood cell
RCF	Relative centrifugal force
r.p.m.	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SOP	Standard operating procedure
SPF	Specific-pathogen-free
US	United States of America
UV	Ultraviolet
V4	Live, thermotolerant, avirulent vaccine against Newcastle disease
w/v	Weight per volume (for instance g/mL or g/100 mL)

1.0

General introduction

1.1 Introduction

The control of Newcastle disease (ND), a major constraint to village chicken production in many countries of the world, presents special challenges to national veterinary authorities and farmers. Although effective vaccines are available from large pharmaceutical companies, they are rarely used in areas where high environmental temperatures, lack of a stable infrastructure, small flock sizes and shortage of foreign exchange are commonplace. In countries where village chicken farmers who wish to protect their flocks against ND are required to buy vaccine, the cost of the vaccine is an additional constraint. The more expensive the vaccine, the fewer the farmers who will be able to afford to buy it and the more limited the vaccination coverage.

Live, thermotolerant avirulent I-2 vaccine against ND was developed to help overcome some of these problems. The I-2 ND virus strain is free of commercial ownership and is available to government vaccine production laboratories and other relevant agencies in developing countries that wish to produce vaccine locally. The small-scale production of I-2 vaccine for use in village chickens is seen as an interim strategy. The ready availability and use of an affordable, effective means of controlling ND results in an increase in village chicken production. With increased production, the purchasing power of chicken owners will increase, production methods will improve and then more expensive vaccines may come into use.

Thermotolerance

In this manual, the term thermotolerance is used to describe the ability of I-2 ND vaccine and the parent virus to retain a level of infectivity after exposure to heat. For I-2 ND vaccine it is defined by the length of time the vaccine will retain an infectivity titre sufficient to induce a protective immune response, at a particular temperature.

To provide the information needed by vaccine users, vaccine producers should measure the infectivity titre of the vaccine after exposure to a range of temperatures (for instance 4°C, room temperature, 37°C) for realistic time periods (one year, months or weeks, and days respectively).

This manual describes the procedures needed to produce and test live, thermotolerant avirulent I-2 vaccine against ND. It also discusses the handling of samples for confirmation of diagnosis of ND and the procedures used in conducting challenge trials with the vaccine.

The manual is intended for laboratory technicians and scientists who need a step-by-step guide to I-2 ND vaccine production and/or testing. The information presented in this manual should assist national veterinary authorities interested in controlling ND in village chickens to decide if available resources and facilities will support local production of I-2 vaccine. It also presents information that will assist vaccine producers to prepare applications for registration of I-2 vaccine.

1.2 How to use this manual

The manual has six sections:

1. an introduction to ND and its control
2. general aspects of laboratory management and maintenance
3. techniques of I-2 ND vaccine production
4. testing of I-2 ND vaccine
5. practical aspects of vaccine distribution, including packaging, labelling, storage and administration
6. diagnosis of ND.

Information on the preparation of reagents and solutions, registration of vaccine, and other useful reference information is presented in Appendixes 1 to 13. Terms used throughout this manual are defined in the glossary in Section 8.

This manual should be used in conjunction with its companion volumes *Controlling Newcastle disease in village chickens: a field manual* (Alders and Spradbrow 2001) and *Controlling Newcastle disease in village chickens: a training manual* (Alders et al. 2002).

1.3 Newcastle disease and its control

Newcastle disease is a highly infectious disease of domestic poultry and wild birds. It is caused by a virus and is widely regarded as one of the most important avian diseases. Although most avian species are susceptible to infection with the virus that causes ND, chickens are the most susceptible to clinical disease. ND was first recognised in Indonesia and England in 1926 (Doyle 1927) and ND viruses are now found world-wide (Aldous and Alexander 2001).

ND, in its highly pathogenic form, is a disease listed in the World Organisation for Animal Health (OIE) Terrestrial Animal Health Code (2011a), and must be reported to the OIE. OIE-listed diseases are diseases with the potential for international spread, significant mortality or morbidity within the susceptible species and/or potential for zoonotic spread to humans. Countries have an obligation to report outbreaks to the OIE. ND is of major importance in both commercial poultry flocks and village chicken flocks where it may cause outbreaks with up to 100% mortality.

Birds infected with ND viruses can show a range of clinical signs depending on the particular causal strain or isolate of ND virus. The age, health and immune status of the host, presence of concurrent infections and environmental conditions will also influence the severity of the clinical signs. Some strains of ND virus cause no clinical signs, while others kill birds rapidly. Strains of ND virus have been divided into five groups or pathotypes on the basis of clinical signs produced in experimentally infected chickens (Beard and Hanson 1984). These pathotypes describe a range of signs and lesions (Table 1) and it may sometimes be difficult to distinguish clearly one from the other.

Table 1 *Pathotypes of Newcastle disease virus*

Pathotype	Description of disease	Clinical signs and post mortem lesions
Viscerotropic velogenic	Acute lethal infection in chickens of all ages	Haemorrhagic lesions in the gastrointestinal tract
Neurotropic velogenic	Acute infection in chickens of all ages; high mortality	Respiratory and nervous signs
Mesogenic	Less pathogenic with low mortality, usually in young chickens	Respiratory and nervous signs
Lentogenic	Mild, inapparent infection; deaths confined to young chickens	Respiratory signs
Asymptomatic enteric (Avirulent)	Avirulent infection; no mortality	No signs or lesions

ND is caused by a virus called *Avian paramyxovirus 1* (OIE 2011b). The virus particle consists of an assembly of material composed of single-stranded ribonucleic acid enclosed in a protein coat (Figure 1). This assembly is surrounded by an envelope, which is derived from the membranes of the host cell. Projecting from the envelope is a fringe of glycoprotein spikes. These are the haemagglutinin neuraminidase (HN) and fusion (F) glycoproteins that are coded by the viral genome.

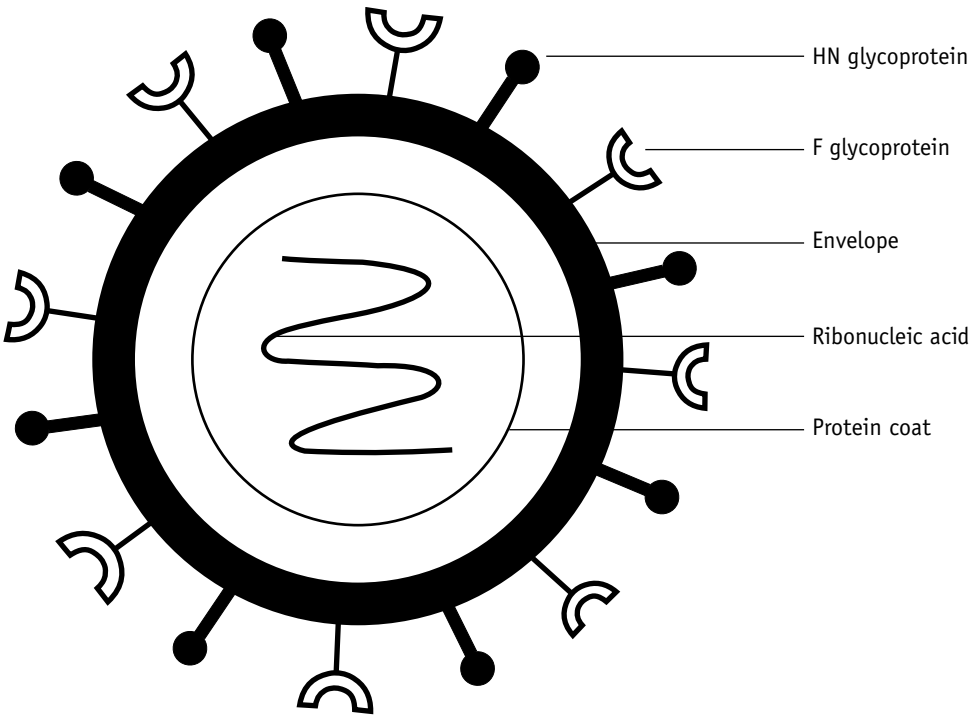


Figure 1: *Newcastle disease virus — a diagrammatic representation.*

In order to reproduce itself (replicate), the ND virus particle must enter a cell. The HN protein assists the virus particle to attach to specific sites on the host cell called receptors, and the F protein helps the envelope of the virus particle to fuse with the cell membrane. This allows the internal constituents of the virus particle to enter the cell cytoplasm, where replication occurs. After replication the virus particle buds from the cell surface and escapes from the cell. This process is shown diagrammatically in Figure 2. The envelope of the virus particle is formed from the membrane of the host cell. Some of the tests described later in this manual exploit the properties of the HN protein.

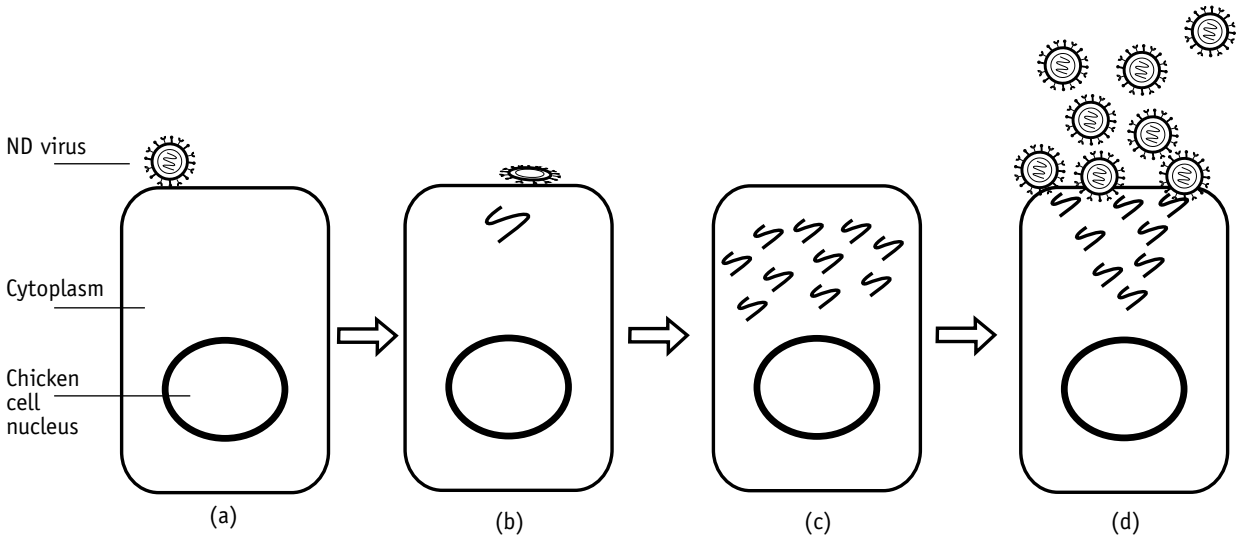


Figure 2: *Replication of the Newcastle disease virus: (a) the HN protein assists the virus particle to attach to a chicken cell; (b) the F protein helps the envelope of the virus particle to fuse with the cell membrane and the internal constituents of the virus particle enter the cell; (c) replication occurs in the cytoplasm of the cell; (d) the virus particle buds from the cell surface and escapes from the cell.*

In many countries ND is controlled by vaccination. Immunogenicity, type of vaccine (inactivated or live) and safety are the main factors governing the choice of vaccine (Alexander 2000). Several different vaccines of appropriate immunogenicity and safety are available for the control of ND in village chickens and the advantages and limitations of various vaccines have been reviewed by Bell (2001). Factors which need to be considered when choosing a vaccine include transportability, cost, prior experience with use of vaccines, structures of existing veterinary services, population distribution and communications infrastructure.

Veterinary authorities wishing to use vaccines to control ND in village chickens must consider available resources, the demand for the vaccine and the willingness of farmers to pay for the vaccine. They must then decide on the option best suited to these circumstances (Table 2). The price of imported vaccine must be weighed against the costs involved in producing a vaccine of an acceptable quality locally. Locally produced 'wet' I-2 ND vaccine will probably be the cheapest, then locally produced freeze-dried I-2 ND vaccine followed by imported NDV4-HR vaccine.

Table 2 *Comparison of Newcastle disease vaccines*

	Newcavac	ITA-NEW	Komarov	La Sota	NDV4-HR	I-2
Type	Inactivated	Inactivated	Live mesogenic	Live lentogenic	Live avirulent	Live avirulent
Immunogenicity	Very good	Very good	Good	Moderate	Moderate	Moderate
Thermotolerance	Moderate	Moderate	Poor	Poor	Very good	Very good
Production	SPF eggs	SPF eggs	SPF eggs (sometimes)	SPF eggs (sometimes)	SPF eggs	Eggs from minimal disease flock
Foreign exchange required	Yes	Yes	Yes, unless produced locally	Yes, unless produced locally	Yes	No
Route of administration	Injection	Injection	Injection	Eye-drop, drinking water	Eye-drop, feed, drinking water	Eye-drop, feed, drinking water
Transmissibility	n.a.	n.a.	Yes	Yes	Yes	Yes

n.a. Not applicable.
SPF Specific-pathogen-free.

If a government institution is to produce vaccine locally, a long-term commitment of staff, facilities and funds is needed to establish and maintain production. Mechanisms for cost recovery (for example, a revolving fund) must be established so that proceeds from the sale of the vaccine are returned to the producer to enable timely purchase of eggs, reagents and consumables needed for ongoing vaccine manufacture.

Another benefit to veterinary authorities is that local production also facilitates the strengthening of local capacity to test imported vaccines to ensure that they meet the national quality standards.

Control of ND by vaccination should always be complemented by good husbandry, hygiene and biosecurity. Good feeding and housing will improve the ability of the chickens to mount a strong immune response to the vaccine. Care should be taken to limit the spread of ND from infected birds by control of the movements of people and animals, segregation of sick birds and correct disposal of infected birds and their remains. Remember that vaccinated birds exposed to virulent ND virus may become infected and excrete virulent virus, although they still remain clinically healthy. Such birds may therefore be a source of infection for unvaccinated birds.

1.4 I-2 ND vaccine

Since 1984 the Australian Centre for International Agricultural Research (ACIAR) has supported projects leading to the development of simple and reliable methods of vaccinating village chickens against ND. Two vaccines with enhanced thermotolerance, NDV4-HR and I-2, were developed for this purpose. NDV4-HR was successfully tested in Asia and Africa and became a commercial vaccine, with the master seed virus under commercial ownership. I-2 ND vaccine is similar to NDV4-HR but is free of commercial ownership, and the master seed virus is available to laboratories in developing countries wishing to produce the vaccine locally.

Strain I-2, an avirulent Australian ND virus isolate, was identified after testing of forty-five isolates of avirulent ND virus. These isolates were examined for antigenicity, safety and ability to spread (Spradbrow, Mackenzie and Grimes 1995).

Eighteen selected isolates were then tested for thermotolerance, and isolates showing enhanced heat resistance were selected. I-2 gave the best results and was grown in eggs from a minimal disease flock to form a master seed. The master seed was then tested for safety and for freedom from bacterial contamination (Bensink and Spradbrow 1999). Subsequent testing confirmed that the master seed is free from extraneous viruses, and bacterial and fungal contamination (Australian Animal Health Laboratory specimen testing report SAN: 02-0506, 2002). Complete test results are given in the I-2 ND vaccine master seed information sheet. See Appendix 13 for contact details.

I-2 ND vaccine has similar properties to NDV4-HR vaccine. It:

- is live
- is thermotolerant
- is not virulent for chickens
- spreads between chickens by contact
- provokes an antibody response similar to that achieved with NDV4-HR
- grows to high titre in the allantoic cavity of embryonating eggs
- does not harm chicken embryos inoculated via the allantoic cavity (no embryos died within 148 hours of inoculation, so mean death time could not be calculated).

The safety of I-2 ND vaccine has been confirmed in SPF chicks (Spradbrow et al. 2001). No clinical signs were observed over a three week period in chicks inoculated by eye-drop with vaccine produced from I-2 ND master seed. Chicks were inoculated at day-old and received $10^{6.9}$ or $10^{7.9}$ EID_{50} per bird (i.e. approximately 10 and 100 times the recommended dose).

Like other thermotolerant vaccines, I-2 vaccine will retain a higher infectivity titre for longer if protected from exposure to sunlight, heat and frequent shifts in temperature. As a general guide, freeze-dried I-2 vaccine stored at 4°C should retain a titre greater than 10^6 EID_{50} per dose for at least 12 months, for 8 weeks at 28°C and at 37°C for 2 weeks (Alders et al. 2002). In contrast, liquid I-2 ND vaccine ('wet' vaccine) diluted 1 in 4 with 2% gelatin will retain high titre for over 2 months at 4°C, and for 7 to 14 days at 28°C.

I-2 vaccine can be administered via eye-drop, drinking water and certain feeds (Alders and Spradbrow 2001). Trials in Mozambique showed that farmers preferred eye-drop to other routes of vaccination: more birds vaccinated by eye-drop survived field outbreaks of ND, the vaccine did not need to be given as frequently and vaccination was easy.

I-2 ND vaccine has undergone testing in several countries including Bhutan, Cambodia, Ghana (Amakye-Anim et al. 2000), Myanmar (Hlaing et al. 2000), Senegal, Tanzania and Vietnam. In laboratory trials I-2 ND vaccine has proven to be protective against local virulent strains of ND virus (Tu et al. 1998; Dias et al. 2001; Wambura, Kapaga and Hyera 2000). In Mozambique I-2 ND vaccine provided approximately 80% protection in the field, in the face of an outbreak, when given every 4 months via eye-drop (Dias et al. 2001).

I-2 ND vaccine produced in eggs from a flock tested and found free from key vertically transmitted poultry pathogens (minimal disease flock) is recommended for the control of ND in village chickens only. It is **not** recommended for use in commercial, small-scale commercial or peri-urban chicken flocks unless more rigorous quality assurance procedures are implemented to ensure the absence of vertically transmitted pathogens of importance in intensively raised flocks.

1.4.1 Characteristics of I-2 ND master seed virus

Bensink and Spradbrow (1999) reported the following characteristics of I-2 ND master seed virus.

- **Mean death time**
  No deaths occurred over a period of 148 hours in embryos inoculated via the allantoic cavity with doses of virus from $10^{1.7}$ to $10^{8.7}$ EID_{50}.
- **Cytopathic effect in cell culture**
  I-2 ND virus caused cytopathic effect in chicken embryo kidney cell monolayers, with destruction in 4 days.
- **Thermotolerance**
  I-2 ND master seed virus in allantoic fluid survived exposure to 56°C for 2 hours.
- **Preparation and presentation**
  I-2 ND master seed virus was prepared in embryonating eggs obtained from a minimal disease flock kept under conditions of strict biosecurity at the Animal Research Institute, Queensland Department of Primary Industries, Brisbane, Australia in 1994. No aerobic or anaerobic bacterial growth occurred in Tryptic Soy Broth and Cooked Meat Medium inoculated with I-2 ND master seed virus. No *Mycoplasma* spp. were isolated.

I-2 ND master seed virus is supplied as a freeze-dried powder consisting of allantoic fluid mixed with 5% skim milk powder in a glass ampoule sealed under vacuum.

1.4.2 Nucleotide sequence of I-2 ND master seed virus

During virus replication, ND virus particles are produced with inactive, precursor F glycoproteins, termed F0. For the virus particles to be infectious, the F0 must be cleaved into two portions: the F1 and F2 polypeptides. This is brought about by the action of specific enzymes (proteases) in chicken cells and tissues. Infectious virus is produced only when suitable cells containing these enzymes are infected.

The cleavability of the F0 glycoprotein is directly related to the virulence of viruses in vivo (Rott 1979). It has been postulated that the F0 glycoproteins of virulent ND viruses can be cleaved by proteases found in many tissues and organs. Infection with these viruses results in the spread of virus throughout the chicken or embryo, damaging many tissues and organs. In contrast, ND viruses of low virulence are sensitive to trypsin-like proteases only, restricting infection to only certain cell types in the chicken or embryo.

The complete genome of I-2 ND vaccine master seed virus has been sequenced (Kattenbelt et al. 2006). Sequence information can be accessed at GenBank — accession number AY935499 (http://www.ncbi.nlm.nih.gov/Genbank).

The virulence of ND viruses is related to the amino acid sequence of the cleavage site of the F gene, which determines the cleavability of the F0 protein. The OIE (2011b) classifies an ND virus as virulent if it has at least three basic amino acids (arginine, R or lysine, K) in the position of residues 113 to 116 and phenylalanine (F) at 117. Thus, most virulent viruses have the following sequence (↓ indicates the position of the cleavage site):

^{113}R-Q-(R or K)-R-↓-F-I-G^{119}

Most avirulent viruses have the following sequence:

^{113}(R or K)-Q-G-R-↓-L-I-G^{119}

The I-2 ND virus has the following sequence (Kattenbelt et al. 2006; Wambura et al. 2007), indicating that it is an avirulent virus: ^{113}K-Q-G-R-↓-L-I-G^{119}.

1.5 Vaccine quality

I-2 vaccine against ND was developed to meet the special needs of village chicken farmers in developing countries. In order to meet these needs, vaccine producers have a responsibility to produce vaccine that is:

- safe — will not cause local or systemic reactions when used as recommended by the manufacturer
- potent — contains sufficient virus to induce a protective immune response
- effective — will protect chickens from virulent ND
- pure — free of extraneous micro-organisms and material
- easy to use
- affordable.

These features define the quality of the vaccine (Soulebot et al. 1997), which is considered 'the single most important determinant of vaccination success or failure' (Mariner 1997).

To ensure consistent production of good quality I-2 vaccine, the producer must put in place standards and controls covering all aspects of its manufacture and handling. These standards and controls 'define the risk or possibility of producing and releasing a product that is worthless, contaminated, dangerous or harmful' (OIE 2011c), should fit the conditions under which vaccines are being produced and, where possible, comply with good manufacturing practice. Standards and controls should not be so burdensome that farmers are unable to afford vaccine to protect their flocks.

The principles of quality assurance (QA), good manufacturing practice (GMP), and quality control (QC) define the standards and controls that ensure the production of good quality vaccine, and are the foundations of good vaccine production.

QA includes all the arrangements made to ensure that the vaccine is manufactured to a quality appropriate to its intended purpose — in the case of I-2 ND vaccine, the vaccination of village chickens. All aspects of vaccine production and testing (such as the facilities and personnel, procedures and records, starting materials, product testing, labelling, packaging and distribution) are considered. QA ensures that the process of vaccine production is uniform and consistent through production procedures and product testing. It ensures that the process of vaccine production is designed, documented, implemented and furnished with personnel, equipment and all resources.

GMP is that part of QA that ensures that a product is manufactured in a safe, clean environment; by specified methods under adequate supervision, and with effective quality control procedures.

QC is that part of GMP concerned with the taking and testing of samples at each stage of the production process to ensure the safety, purity, potency, efficacy and stability of the vaccine. QC also ensures that the vaccine is not released until it passes these tests. QC alone will not guarantee the quality of the vaccine; it is better and cheaper to prevent problems with vaccine quality through good QA and GMP than to rely on tests on the final product!

The procedures and protocols described in this manual are the **minimum** required to ensure the production of I-2 vaccine of good quality, suitable for use in village chickens. Vaccine production and testing protocols should be revised regularly, and staff should be encouraged to refine and improve procedures so that standards outlined in *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (OIE 2011b, c, e), or other publications such as the *European pharmacopoeia* (EDQM, 2010) or *Manual of ASEAN standards for animal vaccines* (1998) are achieved.

2.0

Laboratory management and maintenance

2.1 The vaccine production laboratory

Although I-2 ND vaccine can be produced in a relatively unsophisticated laboratory using simple equipment, there are certain basic laboratory requirements that must be supplied to ensure the production of good quality vaccine. The demand for I-2 vaccine, that is the number of doses required per year, will also influence the equipment and facilities needed.

2.1.1 Basic laboratory requirements

Equipment and facilities

Separate facilities and equipment should be provided for vaccine production. In some cases vaccine production staff may be expected to share facilities and equipment with diagnostic or research virology sections. This is **not** recommended and increases the risk of contamination of I-2 vaccine with other organisms. Where strict separation of vaccine production facilities from research and diagnostic activities is not possible, it is possible to achieve physical separation of working areas and staff in a way that will enable the production of a good quality vaccine.

The basic equipment needed for I-2 vaccine production includes:

1. egg incubators — two would be ideal (to incubate eggs before and after inoculation)
2. egg-candling lamp
3. draught-free clean room, gas supply and Bunsen burner (or biological safety cabinet) for vaccine production
4. balance for weighing salts, antibiotics and other reagents (electronic, accurate to 0.001 g)
5. pH meter
6. refrigerator or cold room for storage of consumables (antibiotics, red blood cell suspension) and vaccine (4°C)
7. freezer for storage of seed lots (–70°C is best)
8. wash-up facilities and sterilising unit — autoclave or pressure cooker; hot air oven (must reach 160°C)
9. centrifuge
10. vaccine dispenser (may be an automatic syringe)
11. vaccine containers — proven virus friendly
12. general laboratory glassware and consumables (see Appendix 2)
13. freeze-dryer (and suitable bottles, stoppers, seals) if producing freeze-dried vaccine.

Distilled water is needed in I-2 vaccine production to prepare phosphate-buffered saline (PBS) used for dilution of working seed and for the preparation of inoculum and red blood cell suspensions, and is needed in the final rinsing of glassware. It is needed for preparation of solutions for dilution of 'wet' vaccine. Double-distilled water produced in an all-glass still continues to be the most reliable and sustainable system for producing water suitable for use in vaccine production and cell culture (Worrall 1997). Distillation units must be cleaned and descaled regularly.

Electricity is needed to power equipment such as egg incubators, refrigerators and freezers as well as egg-candling lamps, balances, pH meters, water distillation apparatus and ovens. All sensitive equipment must be connected to voltage stabilisers, and essential equipment such as egg incubators, freezers and refrigerators should be connected to a standby generator, if possible.

Reagents and consumables should be purchased from a reputable company and be of the required purity. Analytical reagent grade (AR) is generally a good balance of appropriate quality for vaccine production and price. Chemicals should be stored at the correct temperature and used before the 'Use By' or 'Expiry Date'.

Personnel working in vaccine production must receive adequate training in techniques and procedures, and regular 'in-service' refresher courses. Staff should be employed in vaccine production only. If they also work in a diagnostic or research section, these duties should not be performed when vaccine production is underway. Written staffing structures and duty statements should be available, showing who is responsible for what and who reports to whom.

2.2 Laboratory safety

Some chemicals, reagents, organisms or equipment used in the laboratory may be hazardous to laboratory staff. To minimise the risks to staff, it is important to identify potential hazards. Procedures can then be put in place to ensure that work is carried out in a safe, efficient manner. Laboratory staff should be made aware of the hazards and methods of dealing with them.

Chemicals and reagents

- Request a copy of the material safety data sheet (MSDS) for each chemical from the supplier when placing orders. The MSDS gives information on hazards associated with use of the chemical including fire and explosion hazard data, any special precautions needed when handling the chemical, how to deal with accidents and spills and first aid treatment. The MSDS should be placed in a file and kept in the laboratory for ready reference.
- Store chemicals correctly. Read the directions for use and storage on the label.
- Label all stored solutions with the name of the solution or chemical, the concentration, date of preparation, the name or initials of the person who prepared the solution and any hazard warnings.

Glassware and sharps (scalpel blades, needles, etc.)

- Do not use chipped or cracked glassware.
- Always carry large bottles or flasks in an appropriate basket, or carry them using two hands — one to hold the neck of the bottle and the other to support it from underneath.
- Take care when attaching rubber tube to glass tubing or when inserting glass tubing into rubber stoppers. Glass tubing is easily broken.
- Dispose of broken glass, scalpel blades and used needles carefully. The use of appropriate 'sharps' containers is recommended.

Electrical hazards

- Have electrical equipment inspected regularly.
- Do not use equipment or fittings with bare electrical wires, or poorly insulated or worn electrical leads in the laboratory.

Bunsen burner, autoclaves and hot-air ovens

- Take care when working with a Bunsen burner. Work around, not over, the burner so that sleeves and clothing do not catch fire.

- Turn off the Bunsen burner when it is no longer required.
- Do not use Bunsen burners near flammable reagents.
- Allow glassware and other items to cool before removing them from the oven or autoclave, or use insulated oven mitts when removing hot items from the oven.

Ultraviolet (UV) lights

- Ensure that UV lights are turned off before entering rooms fitted with UV lights or using the biological safety cabinet. Exposure to UV light can cause eye and skin damage.

Fumigants

- Ensure that laboratory staff carrying out fumigation know the correct procedures and are provided with proper safety equipment.
- Ensure that rooms are locked and warning signs are placed on doors when fumigation is in progress.
- Ensure that two persons are present during the preparation for fumigation so that assistance is available if problems arise.

Biological hazards

- Take care when handling ND virus, and wash hands thoroughly after handling materials or samples of virus. Conjunctivitis, oedema of the eyelids and mild, generalised signs of fever, chills and headache have been reported in human infections with ND virus (Alexander 2000).

2.3 Aseptic technique

Micro-organisms such as bacteria, fungi and yeast are everywhere — in the air, on work surfaces, in inadequately sterilised reagents and solutions, on glassware and equipment and even on technicians themselves. During production, I-2 ND vaccine may become contaminated with these micro-organisms.

Simple techniques can be used to reduce the probability of contamination of the vaccine.

- Vaccine production should be undertaken in an area where there is little or no human movement and where no other activity is being carried out.
- Only persons essential to vaccine production should be present during egg inoculation and harvesting of allantoic fluid.
- Persons involved in vaccine production must wash their hands and forearms with soap immediately before entering the vaccine production area. Rings, watches, etc. must be removed, and hands, fingernails and forearms should be scrubbed with a brush.
- Clean (sterile is best) caps, gowns and facemasks should be worn properly, with all hair covered and the nose and mouth covered. Gowns must be laundered regularly.
- Footwear or overshoes provided for use in the vaccine production area should be worn only in the vaccine production room.
- Sterile surgical gloves should be worn and swabbed frequently with 70% alcohol during the procedures.
- Talking should be minimised during vaccine production.
- The work surface should be cleared of unnecessary equipment and swabbed with 70% alcohol before and during vaccine production.

Health and safety in the laboratory

1. Always wear appropriate protective clothing in the laboratory, including safety glasses or eye shields if necessary. Remove protective clothing when leaving the laboratory.
2. Wash hands before and after laboratory procedures, and before eating or smoking.
3. Do not smoke, eat or drink in the laboratory or store food in the laboratory refrigerators or freezers.
4. Plan your work and work in a tidy, logical manner.
5. Always use a pipette filler or bulb. Never pipette by mouth.
6. Take care when handling glassware.
7. Know the hazards of particular chemicals and use them with care. Treat all chemicals and reagents as potentially dangerous. Ensure that the MSDS for each chemical used is available.
8. Clean up all spilled chemicals and solutions using an appropriate neutraliser or disinfectant.
9. Label all stored solutions with the name of the solution or chemical, the concentration, date of preparation, name or initials of the person who prepared the solution and any hazard warnings.
10. Place all contaminated laboratory glassware in disinfectant immediately after use.
11. Laboratory waste materials must be autoclaved, incinerated or otherwise made safe before disposal.
12. Bench tops and work surfaces must be cleaned and disinfected before and after use.
13. Know what to do in case of fire: Where are the exits? Where should I go on leaving the building? How do I raise the alarm? Where is the nearest fire extinguisher or fire blanket?
14. Know whom to contact in case of accidents or emergencies, and where the first aid kit is located. All accidents must be reported to the Safety Officer.

- All glassware and equipment needed for inoculation or harvesting should be placed in the vaccine production room or area before the day's procedures begin.
- All bottles, other glassware, pipette canisters, etc. placed on the work surface should be swabbed with 70% alcohol.
- Equipment and other apparatus to be used should be arranged for easy access. Organise the work so that you do not need to reach over materials that could be contaminated.
- No mouth pipetting is allowed; bulbs or pipette fillers must be used. A cotton plug should be inserted into the top of the pipette before sterilisation to maintain the sterility of the pipette during use. Discard the pipette if the cotton becomes wet.
- If the production room is air conditioned, make sure that the filter is cleaned regularly and the air conditioner maintained correctly.

Remember that you are the most important single source of contamination in vaccine production. During normal activity the adult human sheds 10 000 skin scales per minute, many of which will be contaminated with normal skin microflora.

2.3.1 Using a Bunsen burner

- Adjust the regulator of the Bunsen burner so that a blue flame is produced. This is the stable flame used for heating, but it may be difficult to see in strong light. Therefore, it should be extinguished when it is no longer needed.
- Work close to a Bunsen burner to reduce airborne contamination. The updraught created by the rising hot air of a Bunsen flame reduces the likelihood of particles falling from the air into an open vessel.
- When removing a pipette from a pipette canister, tilt the canister a little before removing the lid so that the pipettes will be near the open end of the canister. Remove the lid and flame the open end of the pipette canister. Shake the canister so that a pipette protrudes and remove it. Flame the open end of the canister again before replacing the cap. This will minimise contamination of the remaining pipettes.
- When opening a sterile flask, bottle or tube, quickly pass the open mouth of the vessel through a flame. This destroys any micro-organisms on the outer surface near the mouth of the vessel. It will also heat the air within the neck of the vessel, thus establishing an outwardly directed airflow. This will reduce the likelihood of microbial contamination.
- Flame the mouth of each vessel immediately after opening and just before replacing the top.
- Caps, lids or plugs removed from bottles should not be placed on the bench during flaming and sampling. They should be removed and held with the smallest finger of one hand. This will minimise the risk of contamination.

2.3.2 Using a biological safety cabinet

ND virus is designated as a Group 2 pathogen and should be handled using appropriate biosecurity procedures and practices (OIE 2011d). A Class I, II or III biological safety cabinet is recommended when there is potential for generating aerosols, when handling large quantities of culture or where there is a real need to protect the biological product.

Biological safety cabinets are of three types:

- Class I cabinets are designed specifically to provide operator and environmental protection and not to give protection to the work being handled.
- Class II cabinets are designed to give operator, product and environment protection.
- Class III cabinets provide the highest degree of containment by complete separation of work and worker.

In a biological safety cabinet the work surface and immediate environment are protected from dust and contamination by a constant, stable flow of filtered air. Biological safety cabinets must always be used in conjunction with good aseptic technique.

Biological safety cabinets are not fume hoods and should not be used when handling volatile or explosive chemicals.

Biological safety cabinets are equipped with a UV light that should be turned on about 10–20 minutes before the cabinet is used. This will sterilise all exposed surfaces in the cabinet. To ensure that crevices in the cabinet are sterilised, it is necessary to swab the work surface with alcohol or another sterilising agent before and after each use. Do not put your hands or face near the hood when the UV light is on as exposure to UV light can damage skin and eyes.

- The cabinet should be placed in an area free from draughts and where human movement is minimal.
- If the cabinet is to be used several times during the day, it is best to allow it to run continuously since this keeps the working area clean.
- Make sure that the UV light is turned off before you begin your work in the cabinet.
- Do not allow your hands or other items to come between an open vessel or sterile pipette and the air filter.
- Minimise the use of open flames or Bunsen burners in the biological safety cabinet. The heat generated by the flame produces air currents that may disrupt the laminar air flow.

2.4 Using and maintaining laboratory equipment

Laboratory equipment is expensive and must be maintained correctly to ensure it performs correctly and has a long life.

- Operating manuals should be available for all equipment, and laboratory personnel should be trained in the correct use of the equipment.
- Surge protectors or voltage stabilisers must be fitted to all sensitive electrical equipment, for example egg incubators or freezers. This will help to protect them from irregularities in the electricity supply.
- Check that all sensitive equipment, for example an electronic balance, is located in an appropriate place (away from sunlight and draughts) and on a stable, level bench.
- A logbook should be available for all large equipment, showing its 'history'. Information such as maintenance carried out and problems encountered should be recorded. Regular maintenance of equipment is cheaper than breakdown and repair. Follow the manufacturer's instructions for cleaning and maintenance.
- Processes that depend on equipment such as autoclaves, hot air ovens, vaccine dispensers and balances should be validated — that is tested to show that the equipment is performing its function in the correct manner.
- The temperature of cold rooms or large freezers should be monitored and recorded regularly and procedures put in place so that any malfunction or breakdown is identified, reported and rectified without delay.
- Equipment such as pipettors and electronic balances should be regularly cleaned and calibrated to ensure best performance.
- If an air conditioner is installed in the vaccine production unit, the filter should be cleaned regularly. The manufacturer's manual will give instructions on how to remove and clean the filter, and recommendations on how frequently this should be done.

2.4.1 Centrifuge

Read the manufacturer's manual for operating instructions specific to your machine. In general:

- Always use tubes or containers of the appropriate size and material in the centrifuge. If necessary use sample holders or cushions in the centrifuge 'buckets' to make sure that the tubes fit well.
- Use identical tubes or containers in the centrifuge.
- Do not overfill tubes or containers, or the sample may spill during centrifugation.
- Balance each pair of tubes or containers. For low speed centrifugation with small sample volumes, accurate pipetting may be sufficient. For larger volumes, use scales or a balance to ensure that diagonally opposite tubes or containers are of the same weight.
- Load tubes or containers into the centrifuge making sure that balanced pairs are placed opposite each other. This will allow the rotor of the centrifuge to rotate evenly.
- Switch off the centrifuge if it vibrates at any time during use and correct the problem.

Centrifuge speed (the speed of rotation of the rotor) is measured in revolutions per minute (r.p.m.). However, a more correct measure of centrifuge function is relative centrifugal force (RCF), the force exerted on the material being centrifuged. RCF depends on the speed of the rotor and the radius of rotation, and is expressed as × *g*, a multiple of the acceleration due to gravity. To convert r.p.m. to RCF (or RCF to r.p.m.) easily, use the conversion scale (known as a nomogram) in Appendix 5.

2.4.2 pH meter

- The pH meter should be calibrated each time it is used, or each morning if used all day, with a standard buffer (of known pH), preferably one closest to the desired final pH. If your pH meter allows two-point calibration, the choice of the second standard depends on the final pH desired. For example, if the final pH desired is 8.5, the standard pH buffers used for calibration should be 7 and 10. If the final pH desired is 5.5, the standard pH buffers used should be 4 and 7.
- Make sure the solution you are measuring is at room temperature, since the pH can change with a change in temperature.

2.4.3 Pipettors

Pipettors are used to deliver accurate volumes of liquid, time after time. Both single channel and multichannel pipettors are available. Always read the manufacturer's instructions before using a pipettor for the first time. Appendix 4 presents a step-by-step guide to using multichannel pipettors. In general:

- Select a pipettor of the appropriate volume range.
- Ensure that the tip is the correct type for the pipettor.
- Press the tips on firmly using a slight twisting motion to ensure correct fit.
- **Never use a pipettor without tips.**
- Calibrate and clean pipettors regularly.
- When using a multichannel pipettor to deliver fluids, check that the fluid levels are the same in each tip.

2.5 Cleaning and decontamination, waste disposal

Knowledge of the susceptibility of ND virus to various chemicals and conditions will ensure that correct procedures are used for cleaning and decontamination of laboratories, disposal of waste materials and transport of samples from the field. ND virus is readily destroyed by exposure to formalin, alcohol, merthiolate, lipid solvents, lysol and ultraviolet light (Allan, Lancaster and Toth 1978). It has a high degree of susceptibility to most disinfectants (Geering, Forman and Nunn 1995) and is also inactivated by pH less than 2 or greater than 11. Heat, for example 56°C for 3 hours or 60°C for 30 minutes, will inactivate field strains of ND virus.

2.5.1 Laboratory

It is recommended that vaccine production be performed in premises dedicated to that purpose. If, however, some sections of the laboratory are used for purposes other than I-2 vaccine production, for example diagnostic or research work or for the production of other vaccines, the laboratory and all equipment must be cleaned and decontaminated before and after vaccine production, using procedures that have been validated. There should be a 'rest period' between production runs of different vaccines.

2.5.2 Equipment and consumables

Used glassware should not be allowed to dry out. Soak it in a suitable sterilising agent such as a chlorine-containing compound for at least half an hour. Overnight soaking is preferred. Then wash the glassware in a detergent that is easy to rinse off and non-toxic. Rinse thoroughly in tap water and deionised or distilled water. Dry in the inverted position (so that water will drain out), wrap appropriately and sterilise.

The method of sterilisation depends on the material to be sterilised. Dry heat sterilisation is suitable for glass Petri dishes, flasks, pipettes and metal objects. All materials should be wrapped in aluminium foil or paper to ensure maintenance of sterility after sterilisation. It is important to arrange the items to be sterilised loosely in the sterilising oven to allow good air circulation. Air is not a good conductor of heat and items that are tightly packed in the oven may not be sterilised properly. Allow the oven to reach sterilising temperature (160°C) and then allow one to two hours for sterilisation. Sterilisation begins once the oven reaches the sterilising temperature. Always allow a cooling down period of up to two hours before the oven door is opened.

Sterilisation by moist heat, as in an autoclave or pressure cooker, is more efficient than dry heat sterilisation. For autoclaving, all glassware and equipment should be wrapped in a strong covering that allows steam to penetrate and is impermeable to dust and micro-organisms. Aluminium foil, brown paper or special autoclave paper are suitable wrapping materials. All living micro-organisms, including bacterial and fungal spores, are killed if autoclaved at 121°C for 20 minutes (Versleeg 1985).

Pressure cookers are used in many laboratories to sterilise small items of equipment and solutions. Only materials that can be penetrated by steam can be sterilised this way. Always ensure that sufficient water is placed inside the cooker and that the material to be sterilised is placed in a basket held above the water by a support. Read the manufacturer's instructions to ensure correct operation of your pressure cooker.

Many laboratories will 'recycle' disposable plastic items such as plastic microtitre plates. In most cases it will not be possible to sterilise these materials using heat.

They should be thoroughly decontaminated, cleaned and dried. If sterilisation is necessary, the cleaned and dried plates should be sterilised in 70% alcohol for 30 minutes, then dried under UV light. Plastic eye-droppers or bottles used for vaccine should be cleaned and dried, sterilised in 70% alcohol for 30 minutes, dried, then rinsed out with sterile distilled water or saline solution immediately before filling.

Heat-sensitive indicator tapes and packaging are widely used to show if materials sterilised by autoclave or dry heat have reached the required temperature. Such indicators should not be relied on as an indicator of sterility in autoclaves since they do not show how long the temperature has been maintained at the required level, nor if the required pressure was attained. Integrator or emulator strips (which have been tested against biological indicators) monitor temperature, time and steam quality and are very useful for day-to-day monitoring of sterilisation. Biological indicators (strips or tubes containing bacterial spores) or enzyme indicators should be used to validate all sterilisation processes.

Further information on sterilisation procedures can be found in Versleeg (1985) and Freshney (1994).

2.5.3 Cleaning egg incubators

Egg incubators should be cleaned between batches of eggs to prevent contamination. Small egg incubators are easily cleaned and disinfected using alcohol or a disinfectant with a broad spectrum of activity.

Equipment and materials

Gauze swabs or cloth
Disinfectant, for example 70% alcohol or commercial disinfectant with a broad spectrum of activity

Procedure

1. Remove all solid waste materials from the inside of the incubator and the racks with a damp cloth or gauze.
2. Swab all surfaces with disinfectant or 70% alcohol.
3. Allow incubator to dry before placing eggs inside.

2.5.4 Fumigating egg incubators and vaccine production rooms

Large egg incubators and rooms used for vaccine production should be cleaned and fumigated regularly. The gas formaldehyde may be used since it is a powerful, low cost, non-corrosive disinfectant. It is virucidal, bactericidal and fungicidal and acts most efficiently on wet surfaces. It is sold as a 40% solution in water called formalin. When formalin is mixed with potassium permanganate, formaldehyde gas is liberated. Heat is generated during this reaction so the chemicals should be mixed in a metal container (not glass or plastic). The container should be deep and at least five times the volume of the liquid since bubbling and splattering occur during the reaction (Bermudez 2003).

Fumigation using formaldehyde should be done under close supervision and only by experienced personnel. Exercise caution when using formaldehyde. It is toxic if inhaled; irritating to the eye, skin and respiratory system and possibly carcinogenic — it must be handled with care. For successful fumigation, the area to be fumigated should be able to be sealed.

Safety precautions

Wear gloves when handling and mixing chemicals.

Wear a suitable respirator or mask.

Place a sign on all doors warning of the presence of formaldehyde gas.

Always work with a second person so that assistance is available if problems arise.

Always add the formalin to the permanganate to avoid splashing.

Since heat is generated during the reaction, the mixing container should be at least 2 m away from combustible materials.

After fumigation is completed, allow vapours to disperse before entering the room.

Equipment and materials

40% formaldehyde solution	— 30 mL per cubic metre
Potassium permanganate ($KMnO_4$)	— 20 g per cubic metre
Water	— 30 mL per cubic metre
Metal container	— at least five times volume of liquid; not glass or plastic

Procedure

1. Measure the length and width of the room or incubator to be fumigated and estimate the height (in metres). Fix the exit door in an open position while preparing and mixing the chemicals.
2. Calculate the volume to be fumigated by multiplying the length by the width by the height. This will give the volume of the room or incubator in cubic metres.
3. Calculate the weight of potassium permanganate and volumes of water and formalin required using the following table:

Volume (m^3)	Formalin (mL)	Potassium permanganate (g)	Water (mL)
0.5	15	10	15
1.0	30	20	30
2.0	60	40	60
3.0	90	60	90
5.0	150	100	150

4. Weigh the required amount of potassium permanganate into a large, deep metal container, at least five times the volume of the liquid.
5. Measure the required volumes of water and formalin into a container and mix. Use a container with a wide mouth that will pour quickly.
6. Close all windows.
7. Pour the diluted formalin over the potassium permanganate quickly but smoothly.
8. Quickly leave the area, closing all doors.
9. Place a warning sign on the exit door and leave closed for 24 to 48 hours.

2.5.5 Waste disposal

Separate bins should be provided for different types of laboratory waste — for example paper, general laboratory waste, biological material and sharps. These bins should be clearly labelled and a hazard label attached if necessary. The contents of the bins should then be disposed of correctly.

In general, all biological materials such as eggs, bacterial cultures, blood and contaminated materials should be autoclaved before disposal. If an autoclave is not available, I-2 ND-virus-infected eggs may be incinerated.

Used syringes, needles and broken glass must be placed into the sharps bin and disposed of safely.

2.6 Keeping track of stocks, reagents and consumables

Planning will decrease the waste of valuable resources and ensure that stocks are available when needed. Do not wait until the last item or part of an item is used before ordering replacements since testing may be required to ensure that new stock or supplies are suitable for use in vaccine production.

Keep an up-to-date list of all reagents and consumables, equipment and suppliers. Include the brand and catalogue number, certificate of analysis, MSDS, information on storage conditions and grade required (for instance, AR or cell culture). This will make ordering easier.

An estimate should be made of the number of doses of vaccine and the dose format (number of doses per vial) required each year so that consumables can be ordered and vaccine can be produced and tested in time to meet demand.

An inventory of vaccines in stock should be done regularly (each three to six months, for example). All information on each batch of vaccine should be written in a vaccines register and the register filled in each time vaccine is prepared or dispensed. Similarly, a register of master seed and working seed stocks should also be kept.

2.7 Record keeping

Good records are a key element in good laboratory management. They provide written evidence that each step in the production or experimental process has been properly planned, monitored and performed. Well-kept records can also assist when problems arise since they will allow the entire process to be reviewed and the fault to be identified.

A laboratory day book is the simplest way to keep records of the day-to-day activities of the lab. This should be a hard-covered exercise book (preferably A4). Before use, page numbers should be written in a prominent position on each page (for example, on the top right hand corner of the page) and the first two or three pages ruled up and set aside as an index. This should be filled in as each activity is completed. Standards and frequently used information should be written in a prominent place, such as inside the front or back cover of the book. Such information could include:

- the minimum field titre of I-2 ND vaccine (10^6 EID_{50} per dose: see 'Minimum titres' in Section 4.4)
- the minimum production titre of I-2 ND vaccine (10^7 EID_{50} per dose: see 'Minimum titres' in Section 4.4)

Ready reckoners are also useful additions to a laboratory day book. For instance, a table of volumes of stabiliser added to the allantoic fluid to stabilise vaccine for freeze-drying could be drawn up and consulted each time vaccine is made.

All procedures should be standardised and documented. When the same technique is used each time vaccine is made or a test performed, you can be sure that the product will be the same and the results can be compared.

- Procedures should be written down in the form of a standard operating procedure (SOP). This gives step-by-step instructions for each part of vaccine production and testing and for any associated procedures (see Appendix 3).
- You should read through the SOP before doing the test, especially if it is some time since the test or procedure has been performed. Do not rely on your memory.
- All calculations and results should be recorded.
- Controls must be included in tests to ensure that the test is working correctly.

Documents and records will probably also be required by the national registration authority responsible for regulating the distribution and use of veterinary drugs and vaccines. These records should be filed and stored for easy access. They include:

- standard operating procedures
- bench records — the laboratory records of day-to-day activities, including the raw data, calculations and any changes to the usual procedures
- batch manufacturing records — all information from the bench records relevant to the particular batch of vaccine
- batch release forms — a summary document giving details of batch number, date of production and release, number of containers produced and results of tests on a batch of vaccine, together with the signature of the vaccine production supervisor approving release of the batch.

Records should be kept of all vaccine distributed and of complaints that are received relating to the use of vaccine. All complaints should be investigated and any action taken must be recorded. A system of recalling batches of vaccine found to be faulty after release should also be put in place.

3.0

I-2 ND vaccine production

3.1 An overview of I-2 vaccine production

The techniques involved in I-2 vaccine production are relatively simple. The I-2 ND virus is inoculated into the allantoic cavity of embryonating chicken eggs at 9 or 10 days of incubation. The virus infects and grows in the cells lining the cavity, and allantoic fluid is harvested 96 hours after inoculation when the virus infectivity titre is high. The fluid is then processed to make I-2 vaccine. Tests to confirm the safety and potency of the vaccine are performed throughout the production process. A flow chart of I-2 vaccine production is shown in Figure 3. Figure 4 repeats the flow chart and shows where each procedure may be found in this manual.

I-2 vaccine may be produced in the 'wet' (liquid) or freeze-dried form, depending on the equipment and staff available. 'Wet' vaccine is cheaper to produce than freeze-dried vaccine since it does not require expensive, specialised equipment and skilled maintenance personnel. On the other hand, freeze-dried vaccine has a significantly longer shelf life. Table 3 compares the important features of wet and freeze-dried I-2 vaccine production, storage and handling.

Table 3 *Comparison of wet and freeze-dried I-2 ND vaccine production*

	Wet I-2 vaccine	Freeze-dried I-2 vaccine
Staff training	Training in small-scale I-2 vaccine production and quality control recommended[a]	Training in small-scale I-2 vaccine production and quality control recommended[a]; trained staff required to operate and maintain freeze drier
Equipment	Cheap to produce — no specialised equipment needed	Expensive equipment needed to freeze-dry vaccine
Vaccine containers	A range of 'virus friendly' containers may be used, including glass and plastic containers	Glass vials, caps and seals are costly and limit cost-effective minimum dose format
Storage space	Requires greater amount of storage space	Less storage space required
Storage time at 4°C	May be stored for only 8 weeks at 4°C with no significant drop in titre[b]	May be stored for over 12 months at 4°C with no significant drop in titre[c]
Cold chain	More stringent and far-reaching cold chain needed — 'wet' vaccine may be stored for up to 2 weeks at 28°C[b]	Less stringent and far-reaching cold chain needed — freeze-dried vaccine may be stored for up to 8 weeks at 28°C with no significant drop in titre[c]
Ease of use	Ready to use — no dilution required in the field	Dilution required in the field

a See Appendix 13 for contact details.
b Depends on the dilution and stabiliser.
c Depends on the stabiliser.

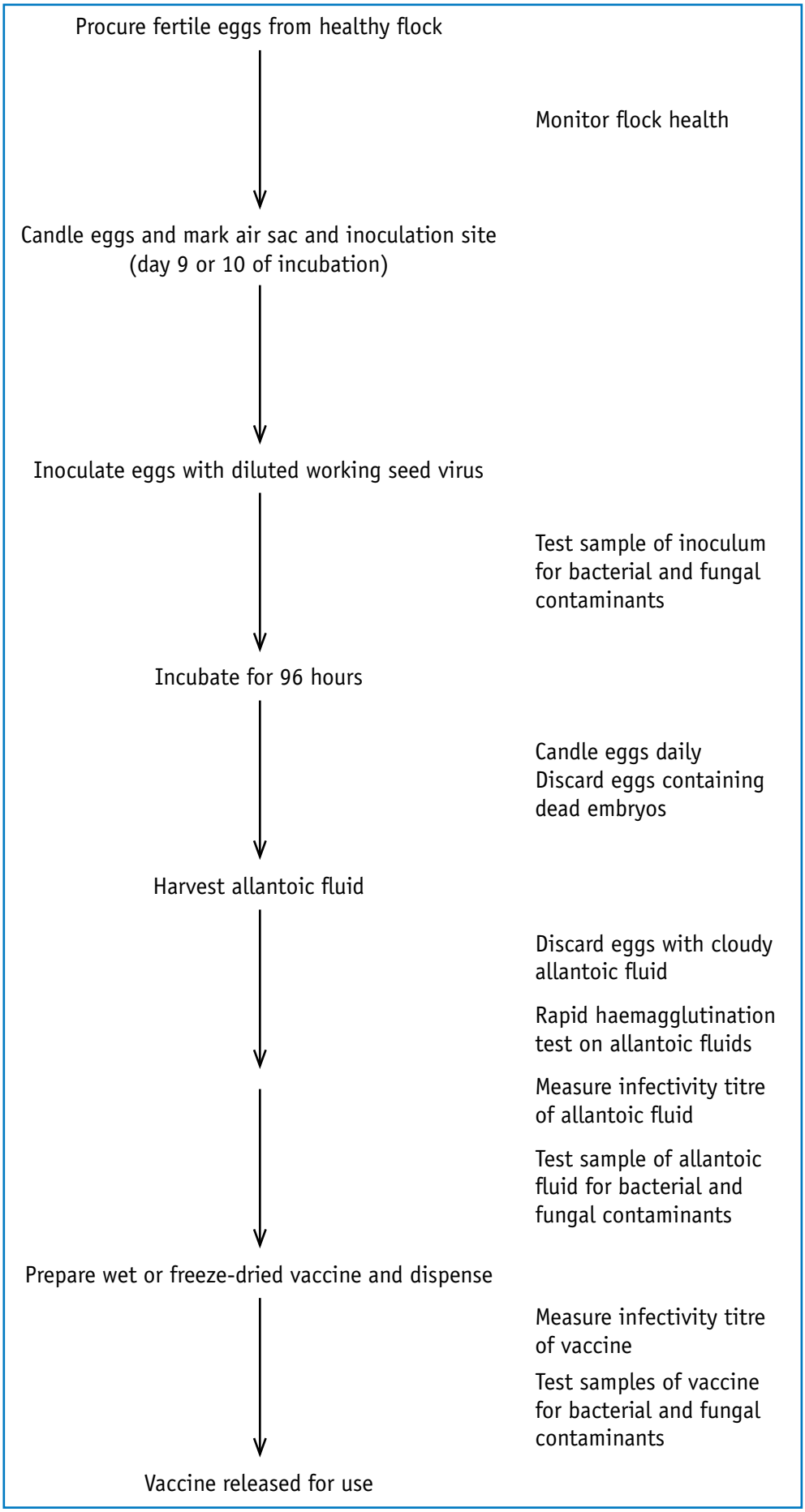


Figure 3: *Flow chart of I-2 vaccine production.*

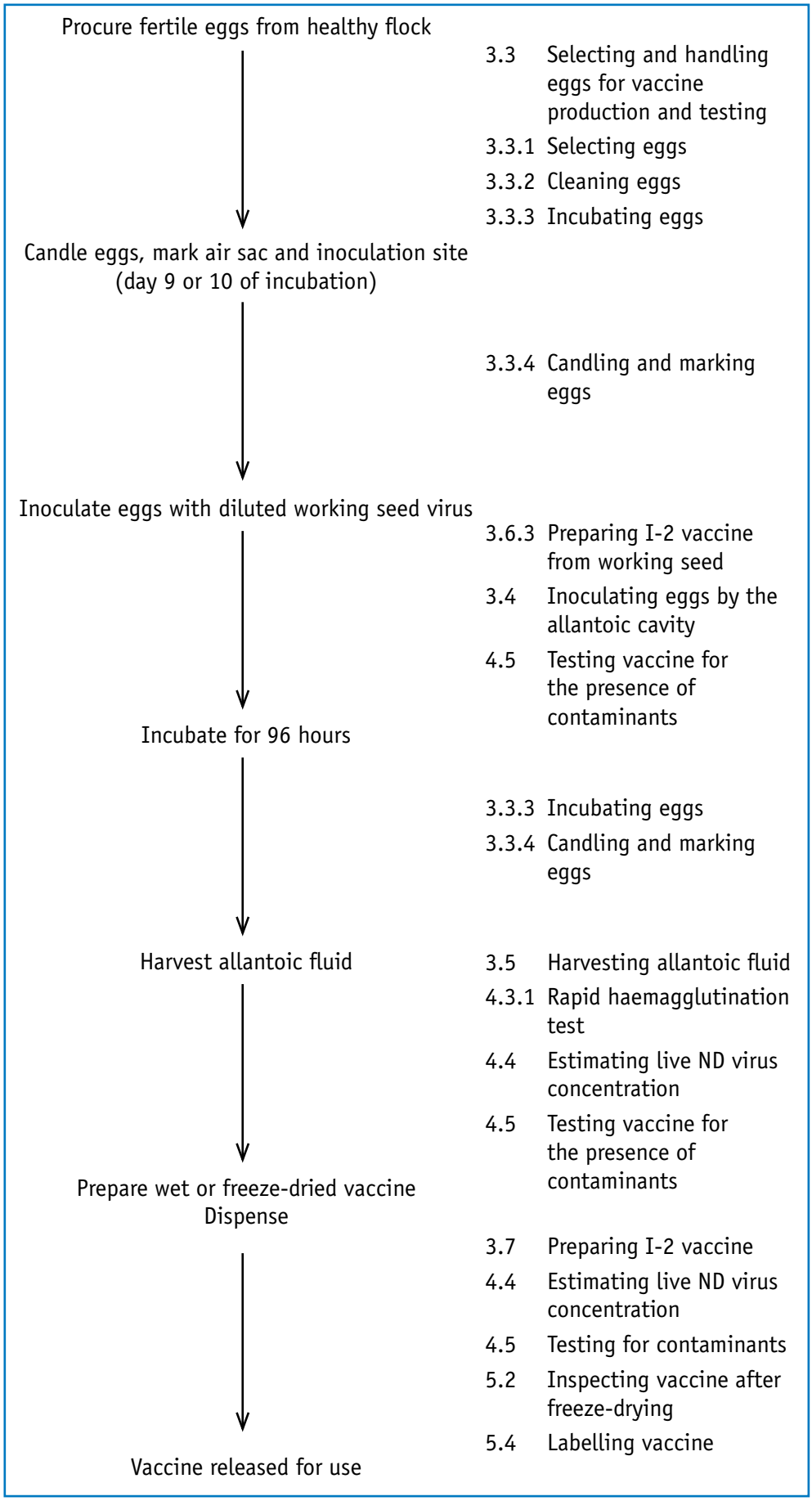


Figure 4: *Flow chart of I-2 vaccine production showing where each procedure may be found in this manual.*

3.2 The structure of the embryonating chicken egg

Embryonating avian eggs have been used as vaccine 'factories' and culture systems for the isolation and propagation of viruses for many years. The hospitable sterile environment and diversity of cell types present in the embryo and its supporting membranes make it ideal for this purpose. The term 'embryonating' is used to identify any egg within which an embryo is developing. Fertile eggs are commonly described as 9 or 10 days old. This refers to the length of time the egg has been incubated, not to the time since the egg was laid.

The structure of the 9- to 10-day-old embryonating egg is shown in Figure 5. The developing embryo is separated from the external environment by the **shell**. Pores in the shell allow air and moisture to enter and leave the egg. It is important to incubate eggs in a humid environment since they will lose moisture if incubated in low humidity and the embryo will eventually die.

The **shell membrane** is the thin, white membrane that lines the inner surface of the shell and is closely attached to it. It helps to exclude micro-organisms from the egg and allows diffusion of gases. It forms the air sac at the blunt end of the egg.

The **air sac** plays an important part in respiration and pressure adjustments within the egg. The air sac is normally found at the blunt or rounded end of the egg.

The **chorio-allantoic membrane** is the membrane just inside the shell membrane. It is attached to the embryo and surrounds it and all the other extra-embryonic membranes. It is formed at around the fourth to the tenth day of incubation by fusion of a layer of the allantois and an adjacent layer of the chorion. It is highly vascular and serves as the respiratory organ of the embryo. This membrane encloses a large cavity: the allantoic cavity.

The **allantoic membrane** is involved in the metabolism of proteins, and the **allantoic cavity** is a reservoir for storing nitrogenous wastes and mediating gas exchange between the embryo and its surroundings. At day 7 it contains approximately 1 mL of fluid, increasing to 5–10 mL at day 13. Allantoic fluid is essentially a physiological salt solution. In the early stages of development the embryo excretes urea and in later stages, uric acid, so that from around day 12, the allantoic fluid may appear slightly milky due to the presence of urates. From day 7 to day 10, the pH of the allantoic fluid is 8.0; at day 18 to 19, the pH is 5.0–6.0.

The **amniotic membrane** encloses the embryo in a sac containing 1–2 mL of amniotic fluid. It secretes and absorbs the amniotic fluid and protects the embryo against physical damage. Amniotic fluid begins to accumulate from around day 5 of embryonic development and initially is a dilute solution of inorganic salts. It reaches its greatest volume of 3–4 mL around day 13 when it contains increased protein.

The **yolk sac**'s primary role is to provide nutrients for the developing embryo. It has an important secondary function in the formation of the first blood vessels and blood cells. Antibodies are present in the yolk of eggs laid by immune hens and are absorbed by the embryo around day 15 of incubation (Senne 1998).

The **albumen** is rich in protein and is essential to the growth of the embryo. It is enclosed in a sac and will decrease in volume but increase in viscosity during the development of the embryo as water and solutes pass into the yolk.

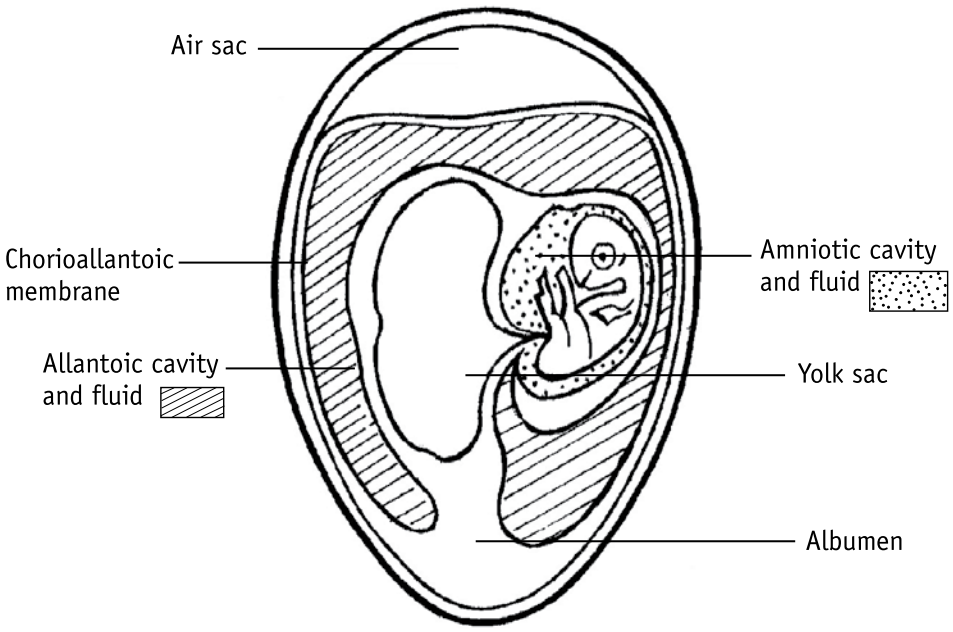


Figure 5: *Structure of the embryonating egg at 9 to 10 days of incubation.*

3.3 Selecting and handling eggs for vaccine production and testing

Fertile eggs used to produce I-2 vaccine must be obtained from healthy flocks.

Where funding is not limiting, it is best to use specific-pathogen-free (SPF) eggs. These are eggs produced by flocks that are kept in strict biosecurity and are shown to be free of specified avian infectious agents by rigorous microbiological and serological monitoring. However, SPF eggs are expensive to produce or purchase, frequently unavailable and problems with embryo viability have been reported (Allan, Lancaster and Toth 1978).

In many countries the costs of I-2 vaccine production will be borne by the end user of the vaccine — the owners of small flocks of village chickens living in rural areas. In these cases, ND vaccine of an acceptable quality can be produced in eggs from flocks that are not SPF. Alders et al. (2002), Buza and Mwamuhehe (2001), Dias et al. (2001) and Tu (2001) report the production and use of I-2 ND vaccine from high quality eggs produced from healthy, minimal disease flocks that are regularly screened for key poultry diseases that can be transmitted through eggs.

It is important that those considering the local production of I-2 vaccine for use in village chickens weigh the benefits (and risks) of using an affordable vaccine of acceptable quality produced in eggs from a minimal disease flock against the losses farmers will experience when ND is not controlled because a vaccine produced in SPF eggs is too expensive for them to purchase.

We strongly recommend that quality assurance managers supervising manufacture of I-2 ND vaccine in small-scale production facilities adopt a risk-based approach to GMP. This will facilitate better and more informed decision-making through identification of potential quality issues and the development of a package of appropriate controls and procedures that ensures the production and distribution of vaccine of acceptable potency (Young et al. 2009).

Eggs used to produce vaccine should be procured from one source to ensure uniformity of production and flock management (a secondary source of good quality fertile eggs should also be identified in case there are breakdowns in health status and production of the principal source of eggs). Records of flock immunisation history, including vaccines used and date of administration; flock health history and flock egg production including numbers of eggs produced and hatchability should be kept. These provide information on the 'quality' of the eggs produced.

To minimise the risk of vertical transmission of key avian pathogens from the parent layer flock:

- Use eggs from clinically healthy birds with a normal pattern of egg production.
- Conduct regular serological tests for *Mycoplasma* spp. and *Salmonella* spp. (to confirm the presence of a protective titre if the flock is vaccinated, or to confirm the absence of the pathogen in a flock that is not vaccinated).
- Have an adequate vaccination program in place to minimise the risks of infection with *Salmonella* spp., *Mycoplasma* spp., egg drop syndrome and infectious bronchitis during the rearing and laying period.

Eggs from flocks that have been vaccinated against ND can be used for I-2 vaccine production. Antibodies against ND will be present in the yolk of eggs laid by immune hens and will be absorbed by the embryo after the fifteenth day of incubation (Senne 1998). However, the antibodies will not be present in the allantoic fluids, and I-2 ND virus inoculated into the allantoic cavity of such eggs at day 9 or 10 of incubation will grow to adequate titre for harvest for vaccine production at day 13 or 14. The presence of antibodies in the yolk will, however, affect the determination of mean death time (MDT). This is discussed further in Section 6.2.

It is possible to store freshly laid fertile eggs for up to one week before incubation is commenced. At temperatures less than 25°C, the embryo is dormant. Storage in a cool room at 10–16°C and relative humidity of 70–80% is best. If a suitable cool room is not available, eggs may be stored in an air conditioned room or, if there is no other option, at room temperature. However, some embryos will not survive. Do not store at 4°C.

3.3.1 Selecting eggs

Procedure

1. Inspect and candle eggs.
2. Discard eggs that:
   - are cracked or broken
   - are poorly shaped
   - are heavily contaminated with faeces or dirt
   - have fragile shells
   - are infertile
   - have a misplaced air sac.

Eggs that are poorly shaped or have rough or thin shells are produced by unhealthy chickens. These eggs are generally of lower fertility and will be easily cracked.

Standard operating procedures for vaccine production and testing

The demand for the vaccine, the existing facilities and staff and whether 'wet' or freeze-dried vaccine is manufactured will determine the volume of I-2 ND vaccine produced and the equipment and materials needed. Therefore no attempt has been made to give a detailed list of equipment and materials for each procedure described in this manual.

A general list of the equipment, glassware and consumables needed in the production and testing of I-2 vaccine is given in Appendix 2. Using this list, the staff of each vaccine production unit must write standard operating procedures (SOPs) appropriate to their needs. Therefore:

- Read through the procedure.
- Estimate your glassware, equipment and consumable needs.
- Prepare a list of glassware, equipment and consumables suited to your needs.
- Prepare an SOP for each aspect of vaccine production and testing.
- Review and revise the SOP after you have gained experience with vaccine production.

3.3.2 Cleaning eggs

It is important to clean eggs before they are placed in the incubator since the shells may be contaminated with faeces, feathers or dust. These may carry micro-organisms, some of which could contaminate vaccine and be pathogenic to chickens.

Wipe the eggs with gauze moistened with 70% alcohol. Eggs may also be dipped in antiseptic solutions such as 0.1% Chloramine B solution (Tu, pers. comm.) or fumigated.

3.3.3 Incubating eggs

The development of the chicken embryo depends on its environment. In the laboratory, the technician can control the environment of the embryo by regulating the temperature, humidity, frequency of egg turning, egg orientation and gaseous environment. Of these factors, the most critical is temperature.

The optimum temperature for incubation of chicken eggs is 37.5°C (99°F) dry bulb temperature or 31.5°C (88°F) wet bulb temperature. Embryos will tolerate temperatures lower than this, but embryonic development will be slower. In contrast, the developing embryo is very sensitive to high temperatures. Embryos from 1–5 days of incubation are very sensitive to heat and will not survive exposure to temperatures of 40–43°C. Five-day-old embryos will be killed by temperatures of 45–47°C.

Procedure

1. Turn on the incubator and allow it to warm before use. Eggs awaiting inoculation should be stored in a clearly marked incubator, separate from inoculated eggs.
2. Place eggs carefully into the incubator racks with the blunt end uppermost. The air sac is located at the blunt end of the egg.
3. Arrange the eggs into rows, using pieces of polystyrene or paper to fill the gaps between rows if necessary. Wedge eggs into the racks so that they will not roll during incubation.

4. Fit the incubator racks into position in the incubator.
5. Ensure that the temperature of the incubator is 37.5°C and humidity is 60–65%. In some incubators the humidity is regulated by placing a dish of water at the bottom of the incubator, or at the top of the incubator near the bulb and fan. If fans are fitted inside the incubator, check that they are working. If bulbs provide the heating, check that these are also working.
6. Turn eggs through 90° as frequently as possible (or hourly if the incubator will turn the eggs automatically). Try to turn the eggs an odd number of times each day so that they rest on different sides each night. Once eggs have been inoculated there is no need to turn them.
7. Each day check the temperature of the incubator and that it is working correctly.

NOTE:

- Do not turn eggs once they have been inoculated.
- If the electricity supply is disrupted during incubation and embryos cool, embryonic development will be affected.

3.3.4 Candling and marking eggs

Eggs are candled to check their fertility, the normal development of the embryos and their viability. During candling a strong light held above or below the egg penetrates the shell and allows the contents to be seen. Candling is easiest in eggs with white shells and after 8 or 9 days of incubation.

In vaccine production and testing, eggs are candled before inoculation, during incubation and before the harvest of allantoic fluid. Before inoculation, eggs are candled to ensure embryos are alive and so that the 'landmarks' that act as a guide to the site of inoculation of the allantoic cavity can be observed and marked. During the incubation of inoculated eggs, candling is done to monitor embryonic development and survival.

Candling is best carried out in a warm, darkened room. A simple candling lamp may be improvised from a torch (or flashlight). In our experience, the method described below has given the best results:

Procedure

1. Turn on the candling lamp.
2. Hold the blunt end of the egg against the soft socket (aperture) of the candling lamp.
3. Rotate the egg to locate the:
   - embryo
   - air sac
   - blood vessels
   - yolk sac.

   Ensure that the embryo is an appropriate size for the stage of incubation and is healthy (a healthy embryo moves in response to the warmth of the candling lamp).
4. Discard eggs containing weak or dead embryos.
5. Choose a clear area away from major blood vessels, the yolk sac and the embryo. Mark the edge of the air sac on the shell using a pencil.
6. Mark the site of inoculation 2–3 mm above the edge of the air sac.
7. Replace the eggs in their racks, air sac uppermost and return them to the incubator.

8. Record the number of living embryos and the total number of embryos examined at each time period.

NOTE:

- The candling lamp may be hand-held or clamped in a laboratory stand.
- Eggs with white shells are easier to candle than eggs with brown shells.
- If a large number of eggs are to be candled, do not take them from the incubator to the candling area all at once since they will cool when out of the incubator. Embryonating eggs at 9 days of incubation can usually tolerate up to one hour out of the incubator without any effect on viability.
- Eggs containing healthy embryos will have an orange-yellow colour due to the presence of blood vessels and circulating blood. The blood vessels will be seen easily and the embryo should move in response to the warmth of the candling lamp.
- In eggs containing dead embryos, the blood vessels will be thinner or absent and less easy to see.
- Learners may prefer to mark other landmarks (for example, the position of vessels) on the shell as well.

3.4 Inoculating eggs by the allantoic cavity

The embryonating chicken egg provides an ideal environment in which to grow viruses. Lentogenic strains of ND virus and avirulent strains such as I-2 grow readily in the endodermal cells lining the allantoic cavity (Nagai et al. 1979). Inoculation into the allantoic cavity is relatively easy and is best done on day 9 to day 12 of incubation. The volume of the inoculum used is 0.1–0.2 mL, and large volumes of virus-infected fluids can be harvested after incubation of the inoculated eggs.

Eggs are inoculated by this route for production of I-2 ND virus working seed from master seed, for vaccine production, measuring the infectivity titre of virus and isolating and characterising ND virus. For I-2 vaccine production, inoculation is generally performed at 9 or 10 days of incubation.

Inoculation must be performed using aseptic technique.

Procedure

1. Remove the eggs from the incubator; pack them into trays with the blunt end (air sac) uppermost. If a large number of eggs are to be inoculated, do not take them from the incubator to the candling area all at once.
2. Transfer the eggs to the candling area. Try to keep them warm (37°C) whilst out of the incubator.
3. Candle the eggs to determine viability and mark the site of inoculation using a pencil (see Section 3.3.4).
4. Prepare the inoculum. For vaccine production, use working seed virus (see Section 3.6.3); for preparation of working seed, use master seed virus (see Section 3.6.2).
5. Swab the inoculation site on the egg with disinfectant. Allow to dry.
6. Disinfect the drilling instrument with 70% alcohol.
7. Drill or punch a small hole through the shell at the marked site of inoculation. Take care not to rupture the shell membrane.

8. Attach a 25 G × 16 mm needle to a 1 mL syringe and fill the syringe with inoculum (each needle and syringe should be used to inoculate 30–60 eggs only).
9. Insert the needle through the hole in the shell and angle the syringe and needle as shown in Figure 6. If using a 16 mm needle, insert the needle to its full depth. If using a longer needle, insert the needle only 14–16 mm.
10. Inject 0.1 mL of the inoculum into each egg.
11. Seal the site of injection on the shell (options are given below).
12. Place inoculated eggs in the incubator and incubate at 37 to 37.5°C and 60 to 65% humidity for 96 hours. The incubator should be clearly marked 'I-2 inoculated eggs' and kept separate from the incubator used for eggs before inoculation (where only one incubator is available, it must be cleaned with disinfectant, swabbed with alcohol and allowed to dry before the inoculated eggs are returned to it for incubation).
13. Test the remainder of the inoculum for bacterial and fungal contaminants.
14. Candle the eggs every 24 hours and examine for viability of the embryo. Discard eggs containing embryos that die in the first 24 hours. If embryonic death occurs after 24 hours in eggs inoculated with I-2 ND virus, eggs should be tested for bacterial contamination.
15. Record numbers of viable embryos at each examination.

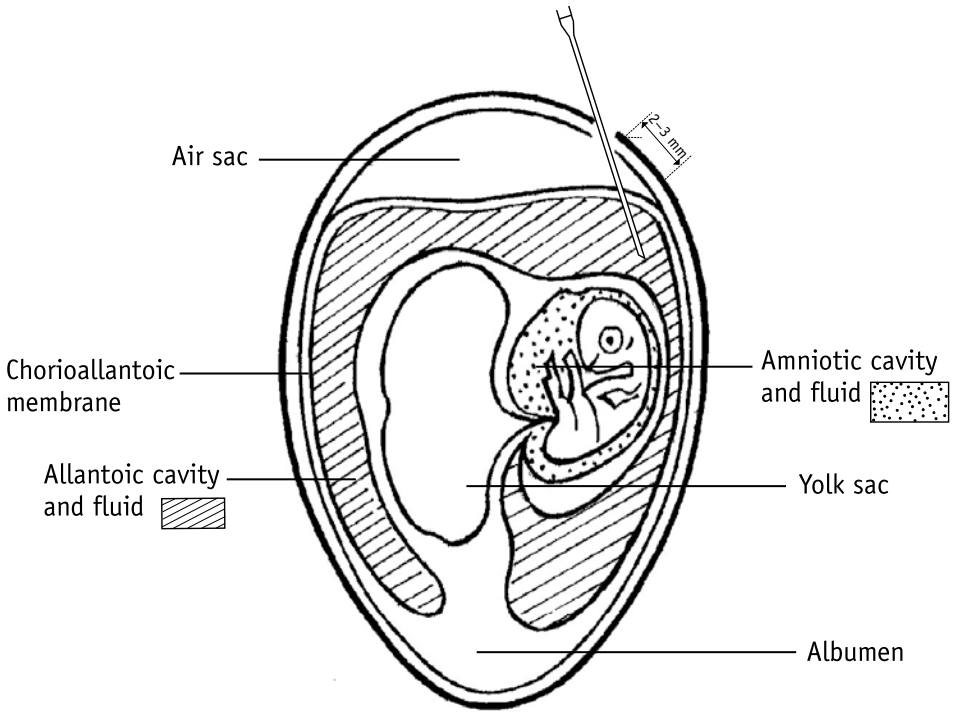


Figure 6: *The 9-day-old embryonating egg showing the position of the needle for inoculation into the allantoic cavity.*

NOTE:

- Instruments that may be used for making the hole in the shell include a needle mounted in a rubber stopper, with around 1 mm of the needle tip exposed; a piece of copper, curved to fit the shape of an egg with 1 mm spike protruding from its centre; and a dental drill or a sharpened punch.
- 25 G × 16 mm (⅝") sterile disposable needles are best for inoculation. Use one syringe and needle for each 30 to 60 eggs.

- Eggs may be sealed with adhesive tape, melted candle wax, Colloidion Flexible (BDH catalogue number 33041), or a mixture made of two parts of solid paraffin (melting point approximately 54°C) and one part of petroleum jelly (Vaseline). Take care if using Colloidion Flexible since it is extremely flammable. Do not breathe the vapour. If using a paraffin/petroleum jelly mixture, keep it melted on an electric heating plate (60°C) and apply to the eggs using a short glass pipette with rubber teat or a cotton wool swab.
- Discard embryos that die in the first 24 hours. These are generally non-specific deaths caused by injury. In eggs inoculated by an experienced operator, up to 2% mortality is considered normal.
- Common causes of embryonic death include (1) embryos of poor viability, (2) haemorrhage due to poor candling and inoculation technique and (3) bacterial contamination.
- If more than 2% of embryos inoculated with I-2 ND virus have died, especially after the initial 24 hours of inoculation, test for bacterial and fungal contamination.
- I-2 ND virus inoculated into the allantoic cavity has no harmful effect on embryos for up to 148 hours — well above the 96 hours required for vaccine production.

3.5 Harvesting allantoic fluid

For vaccine production, allantoic fluid is harvested from I-2 ND-virus-infected eggs 96 hours after inoculation, when virus infectivity titre is high. Usually 6–10 mL of allantoic fluid can be collected from each egg at this time (Senne 1998). Allantoic fluid for vaccine production should be collected only from eggs shown to contain haemagglutinin by the haemagglutination test. Do not harvest from eggs containing discoloured allantoic fluid or dead embryos.

Allantoic fluid is also sampled and tested for the presence of ND virus when attempts are being made to isolate virus or when vaccine, or a sample of virus, is being titrated.

Harvesting must be performed using aseptic technique.

Procedure

1. Remove the eggs from the incubator. Candle to determine the viability of the embryos.
2. Discard eggs containing dead embryos if harvesting the allantoic fluid for I-2 ND vaccine production.
3. Place the eggs in the refrigerator at 4°C for at least 4 hours. This will kill the embryo and cause the membranes to collapse away from the shell. It will also coagulate the blood. The presence of red blood cells in the allantoic fluids may significantly reduce the titre of the virus (Senne 1998).
4. Remove the eggs from the refrigerator.
5. Swab the surface of the eggs around the inoculation site with disinfectant. Allow to dry.
6. Cut and crack the shell around the inoculation site to expose the shell membranes. This may be done using a special shell cutter, cautery or surgical scissors. The shell should be removed to within 5 or 10 mm of the base of the air sac.
7. Remove the shell using sterile forceps and expose the membranes. Place the forceps in alcohol and flame between uses.

8. Inspect each egg before withdrawing allantoic fluid. Discard eggs that have dark and discoloured contents, or discoloured allantoic fluid.
9. Withdraw one drop of allantoic fluid from each egg through a small hole made in the membrane. Use a separate sterile glass or plastic pipette for each egg.
10. Perform the haemagglutination test to confirm the presence of ND virus (see Section 4.3).
11. Discard eggs where haemagglutination has not occurred.

NOTE:

- If harvesting the allantoic fluids for vaccine production, allow the eggs to reach room temperature. Condensation, which forms on the shells of eggs just removed from the refrigerator may cause contamination of harvested allantoic fluid.
- The allantoic fluid from some normal eggs may appear slightly milky. This is due to the presence of urates, which accumulate from around day 12 of incubation. It is important to distinguish normal milky fluids from fluids that are discoloured due to bacterial contamination.

3.6 Vaccine seed lots

I-2 ND master seed virus was prepared from strain I-2, an avirulent Australian ND virus isolate (see Section 1.4). Selection of I-2 ND virus as a suitable master seed virus involved a number of steps:

1. testing of 45 isolates for antigenicity, safety and ability to spread
2. testing of 18 selected isolates for thermotolerance
3. heat treatment of I-2 virus strain
4. growth of thermotolerant I-2 virus strain in eggs from a minimal disease flock
5. testing for safety and for freedom from bacterial, viral and fungal contamination
6. preparation of working seed
7. preparation and testing of vaccine prepared from the working seed.

ACIAR I-2 ND master seed containing thermotolerant, avirulent, antigenic I-2 ND virus is supplied in a glass ampoule in freeze-dried form to laboratories wishing to produce I-2 vaccine. To preserve the infectivity of the master seed it is best stored at –70°C. Try to store the master seed in more than one location so that stocks will not be lost if there is an equipment breakdown or electricity failure. If possible, these locations should have separate electricity supplies and separate backup generators.

I-2 ND vaccine is produced from parent I-2 ND master seed virus using a seed lot system. This process is shown diagrammatically in Figure 7. The I-2 ND master seed virus is reconstituted in PBS and a small amount of the I-2 ND master seed virus suspension is inoculated into embryonating eggs. The allantoic fluid from these eggs is harvested after 96 hours incubation and is used to prepare working seed I-2 ND virus. Many aliquots of working seed virus can be produced from one ampoule of master seed. When a batch of vaccine is prepared, one aliquot of working seed I-2 ND virus is diluted and a small amount of the suspension is inoculated into embryonating eggs. Allantoic fluid from these eggs is harvested to produce I-2 ND vaccine. From one aliquot of working seed I-2 ND virus, one batch of I-2 ND vaccine consisting of many doses is produced.

Using the seed lot system, the number of egg passages from the parent I-2 ND master seed virus to I-2 virus in the vaccine is minimised. This assists in maintaining uniformity and consistency in production (OIE 2011c) and lessens the possibility of genetic change of I-2 virus, which may lead to changes in thermotolerance, virulence, antigenicity and vaccine yield. When vaccine is prepared in the way described above, it is only two passages away from the parent I-2 ND master seed stock virus.

The I-2 ND master seed virus will be accompanied by an information sheet giving information on the master seed, the infectivity titre around the time of dispatch, and instructions for use, based on the procedures set out in Sections 3.6.1 and 3.6.2. Master seed virus is a valuable resource, and when laboratory staff have gained more experience in the techniques of vaccine production, they may wish to revise these procedures to maximise the use of I-2 ND virus seed.

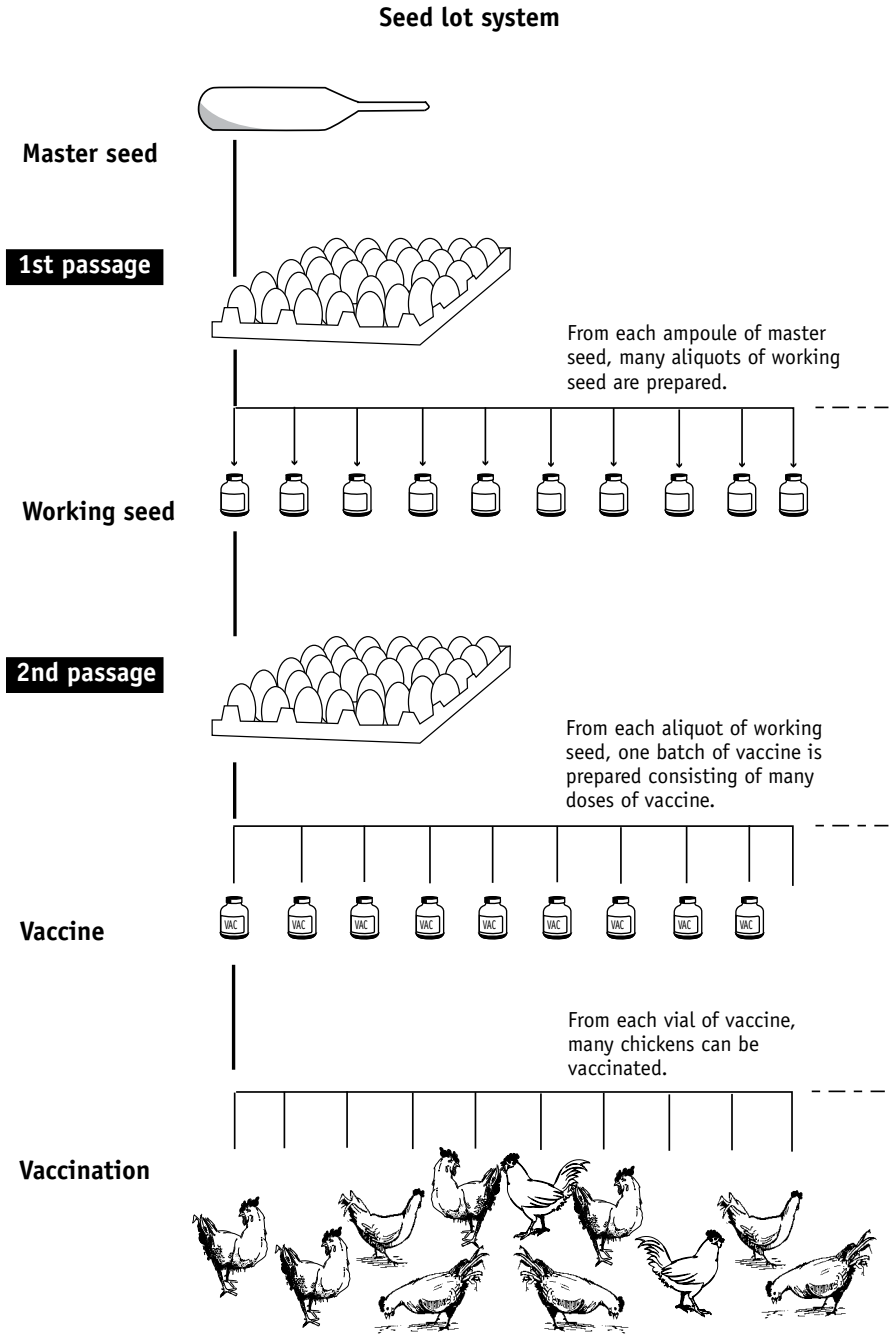


Figure 7: *The vaccine seed lot system.*

3.6.1 Diluting I-2 ND master seed

All procedures must be performed using aseptic techniques.

Procedure

1. Swab the outside of the I-2 ND master seed virus ampoule with 70% alcohol.
2. Open the ampoule carefully and add 1 mL sterile, distilled water to the contents. Mix well.
3. Remove the contents of the ampoule and place in a sterile tube or small conical flask. Add 19 mL sterile PBS. Mix well.
4. Dispense the diluted master seed virus into 1–2 mL aliquots in sterile, screw-capped bottles or cryotubes. Seal and label each aliquot.
5. Place in the upright position in a labelled box or other container and store in a –70°C freezer.
6. Titrate one aliquot of I-2 ND vaccine master seed virus (see Section 4.4). Harvest the fluids from eggs used in the titration that are positive on the haemagglutination test, dispense into aliquots and store as I-2 ND vaccine working seed virus.
7. Label the aliquots, writing the date of production of the working seed and 'I-2 ND vaccine working seed' on the label.

NOTE:

- The titre of the I-2 ND master seed virus should be measured shortly after it is received at the laboratory to confirm that the master seed virus has been handled correctly during dispatch and storage. This information should be recorded in an I-2 ND master seed file, together with all other information, such as date of receipt, storage conditions, passage history and date and results of production of I-2 ND working seed. This information will be required by the national registration authority.
- As 'insurance' against equipment breakdowns or power failures, store some aliquots of master seed and working seed virus in the freezer of another institution (such as a medical research laboratory). Ensure that it is connected to a functioning backup or standby generator.
- Frequent, slow cycles of freezing and thawing are harmful to viruses in suspension. Thaw aliquots rapidly and do not refreeze unused aliquots.
- You may wish to 'colour code' viral seed stocks to assist in identification. For instance, I-2 ND master seed virus could be identified with a blue-coloured label, and I-2 working seed virus could be identified with a green-coloured label.

3.6.2 Preparing I-2 vaccine working seed

All procedures must be performed using aseptic technique.

Procedure

1. Thaw one aliquot of I-2 ND vaccine master seed. Swab the outside of the vial with 70% alcohol.
2. Calculate the volume of inoculum needed. For example:

   30 eggs × 0.1 mL per egg = 3 mL inoculum
3. Dilute thawed I-2 ND vaccine master seed in the calculated volume of PBS. Mix well.
4. Inoculate 0.1 mL of diluted I-2 ND master seed into the allantoic cavity of 9- to 10-day-old embryonating eggs.

5. Candle eggs each day. Discard eggs containing dead embryos.
6. Harvest allantoic fluid 96 hours later. Test the allantoic fluid of each egg using the haemagglutination test (see Section 4.3 for procedure) to determine the presence of virus.
7. Dispense the pooled allantoic fluids into aliquots. Label each aliquot and place in a labelled box or other container. Freeze at –70°C if possible. These aliquots are I-2 ND vaccine working seed virus.

NOTE:

- Antibiotics may be added to PBS used to dilute thawed I-2 ND vaccine master seed.
- Each laboratory should determine the volume of the aliquot of working seed to be dispensed. Larger units producing I-2 ND vaccine in large batch sizes may need to dispense 1 mL aliquots, whereas smaller units may prefer to dispense 0.5 mL aliquots.
- It is advisable to produce the working seed in SPF eggs if they are available. Working seed should also be confirmed free of bacterial and fungal agents, and of extraneous viral agents if possible.

3.6.3 Preparing I-2 vaccine from working seed

All procedures must be performed using aseptic technique.

Procedure

1. Thaw one aliquot of I-2 ND working seed virus. Swab the outside of the vial with 70% alcohol.
2. Prepare 10^{-1} and 10^{-2} dilutions of working seed virus in sterile PBS.
3. Calculate the volume of inoculum needed. For example:
   300 eggs × 0.1 mL per egg = 30 mL inoculum
4. From the 10^{-2} dilution of working seed virus, prepare a 10^{-3} final dilution. For example, for 300 eggs add 3 mL of 10^{-2} dilution of working seed to 27 mL of sterile PBS containing antibiotics.
5. Inoculate 0.1 mL of 10^{-3} working seed dilution into the allantoic cavity of embryonating eggs.
6. Candle the eggs each day and discard those containing dead embryos.
7. Harvest the allantoic fluid 96 hours later. Test the allantoic fluid by the rapid haemagglutination test to determine the presence of virus.

NOTE:

- Antibiotics may be added to the PBS used to dilute thawed I-2 ND vaccine working seed.
- When a small number of eggs are being processed for vaccine production, when technicians are in training or when checks on operator technique are required, the allantoic fluids from all eggs should be tested in step 7.
- Experienced operators processing a large number of eggs for vaccine production may prefer to take a random sample of eggs from each tray (10% is recommended) for testing of allantoic fluid. This will save time and minimise the length of time eggs are open to the environment before harvesting of allantoic fluid.

Optimal dose of ND vaccine seed cultures

I-2 ND seed virus is a valuable resource and ways should be found to maximise its use. Trials were undertaken at the John Francis Virology Laboratory, University of Queensland, to determine the minimum dose of I-2 ND vaccine working seed that could be inoculated into eggs to produce vaccine of acceptable titre. Tenfold dilutions of working seed virus were made and doses of virus from 10^1 to 10^5 EID_{50} were inoculated into embryonating chicken eggs. The allantoic fluid was harvested and the infectivity titre for each dilution determined. This work showed that it is possible to inoculate eggs with 10^1 EID_{50} of working seed virus and still get vaccine of acceptable titre. However, to ensure that each inoculated egg produces virus, a higher dose of 10^3 EID_{50} per egg (10^4 EID_{50}/mL) is recommended.

3.7 Preparing I-2 vaccine

I-2 ND vaccine can be produced in either the wet (liquid) or freeze-dried form. A crucial ingredient of both forms of the vaccine is the stabiliser that is added to help preserve infectivity during storage. Many different stabilisers have been tested and the two found most effective are gelatin (final concentration in 'wet' vaccine 0.5–1%) and skim milk (final concentration in freeze-dried vaccine 5–10%) (Allan, Lancaster and Toth 1978; Bensink and Spradbrow 1999). A number of recent papers describe research on alternative stabilisers and methods of formulating I-2 ND vaccine (Wambura 2009a, 2009b, 2011).

All procedures must be performed using aseptic technique.

A. Freeze-dried vaccine

Procedure

1. Harvest the allantoic fluid from eggs using a 20 mL syringe and needle (13–16 G, long) or vacuum pump and needle (or sterile glass Pasteur pipette). You may use a sterile spatula, small spoon or forceps to depress the embryo and yolk sac while aspirating the allantoic fluid.
2. Pour the allantoic fluid into suitable sterile bottles or centrifuge tubes which have been cooled for several hours in a domestic freezer at –20°C.
3. Place the bottles or tubes into centrifuge buckets and balance the buckets. Place the centrifuge buckets in a centrifuge (refrigerated at 4°C is best) and centrifuge at 1000–1500 × *g* for 15 minutes to remove cells and other solids.
4. Remove the bottles or tubes from the centrifuge. Pour the supernatant into a clean, sterile measuring cylinder or sterile centrifuge tubes. Measure and record the volume of allantoic fluid harvested (if a large volume of allantoic fluid has been harvested, pour the fluid into sterile bottles with screw caps).
5. Collect samples of the vaccine for infectivity titration (see Section 4.4) and testing for bacterial and fungal contaminants (see Section 4.5).
6. Place the allantoic fluid in a refrigerator at 4°C until the results of preliminary testing are received. Prepare the stabiliser.
7. Add the stabiliser (if using skim milk, go to step 6.1 on page 45).
8. Dispense the vaccine into sterile glass vaccine vials using an automatic syringe or dispenser. In our experience, 2.5 mL is ideal for 250 dose vials.

9. Using sterile forceps, fit rubber stoppers loosely into the vials to allow the escape of moisture during freeze-drying. Do not insert the stoppers firmly into the vials.
10. After freeze-drying, collect a number of vials for sterility testing and for infectivity titration. This number is determined by the number of vials of vaccine produced (see Section 4.5).

If using skim milk in PBS as the stabiliser:

6.1 Calculate the volume of sterile skim milk required.

6.2 Measure the sterile skim milk into a sterile glass flask.

6.3 Take the flask containing the allantoic fluid from the refrigerator and place in a sterile metal tray. Pack ice around the flask and place the tray onto a magnetic stirrer. Carefully place a sterile magnetic stirring bar into the flask.

6.4 Carefully add the skim milk to the allantoic fluid.

6.5 Switch on the magnetic stirrer and stir gently for 30 minutes.

6.6 Continue with step 8 on page 44.

NOTE:

- Always confirm the sterility of the stabiliser solution before it is added to the allantoic fluid.
- Generally, one part of allantoic fluid is mixed with one part of sterile stabiliser solution.

Antibiotics in vaccine production

Antibiotics should not be used routinely in vaccine production to compensate for poor aseptic technique. They are expensive, sometimes difficult to obtain and add to the cost of production of the vaccine. In addition, many registration authorities encourage the reduced use of antibiotics as preservatives in vaccines, only approving the addition of specified antibiotics at particular concentrations to vaccine diluent to protect from 'in use' contamination.

Since freeze-dried vaccine may be diluted in the field using non-sterile potable water and sometimes is used for two days, antibiotics may be added to prevent 'in use' contamination. 'Wet' vaccine will also benefit from the inclusion of appropriate concentrations of specified antibiotics.

Further information is presented in Appendix 6.

B. 'Wet' vaccine

To prepare wet I-2 ND vaccine, dilution in 1–2% gelatin or 4% skim milk in PBS is recommended. One part of allantoic fluid mixed with one part of sterile diluent (to give a final concentration of 0.5–1% gelatin or 2% skim milk) gives good results.

Procedure

Follow the procedure for producing freeze-dried vaccine from steps 1 to 7.

8. Dispense the vaccine into sterile containers — for example, single-use plastic eye-droppers with a tamper-proof seal.
9. Collect a number of containers for sterility testing and for infectivity titration.

NOTE:

- Allantoic fluid that has been collected and centrifuged may also be filtered. Using a sterile, stainless steel funnel, filter the allantoic fluid through four layers of sterile gauze into a large measuring cylinder.
- The expected titre of allantoic fluid immediately after harvest, is approximately $10^{9.5}$ EID_{50}/mL. Aim to produce vaccine with a minimum production titre of vaccine of 10^{7} EID_{50} per dose (see Section 4.4) to allow for a reduction in the titre during storage, transport and field use of the vaccine.
- If the amniotic sac is accidentally opened during harvesting, amniotic fluid may be collected together with allantoic fluid. I-2 ND virus does not enter the amniotic cavity after inoculation into the allantoic cavity, therefore the amniotic fluid will only dilute the infected allantoic fluid.
- Although test data on I-2 ND vaccine produced at another laboratory may serve as a guide, each manufacturer must test the stability of the vaccine produced in their own laboratory. This is especially important if different stabilisers or diluents are used, if the concentration of the diluent is changed or if the dilution is varied. Stability testing of vaccines is discussed in Section 5.3.

3.7.1 Worked examples

Example 1

Allantoic fluid collected from eggs is diluted 1 to 1 with 1% gelatin in PBS to make 'wet' I-2 ND vaccine. The titre of the vaccine is found to be 10^{9} EID_{50} per 0.1 mL. Plastic droppers that deliver 40 drops per mL will be filled with the vaccine in the laboratory for delivery of the vaccine in the field. One dose is administered in one drop of vaccine.

(a) What is the titre of the 'wet' vaccine per mL? Per dose?

(b) Will this meet the minimum production titre (see Section 4.4)?

Answer

(a) 1 mL = 0.1 mL × 10

Titre of vaccine per mL $= 10^{9}\ EID_{50} \times 10$

$= 10^{9}\ EID_{50} \times 10^{1}$

$= 10^{9+1}$

$= 10^{10}\ EID_{50}$ per mL

The droppers deliver 40 drops per mL. Therefore:

Titre of 'wet' vaccine per dose $= \dfrac{10^{10}\ EID_{50}/mL}{40\ drops/mL} = \dfrac{10^{10}\ EID_{50}/mL}{10^{1.6}\ drops/mL}$

$= 10^{10} \div 10^{1.6}\ EID_{50}/drop$

$= 10^{10-1.6}\ EID_{50}/drop$

$= 10^{8.4}\ EID_{50}/dose$

(b) *This is above the minimum production titre (i.e. 10^{7} EID_{50} per dose) and the batch may be distributed if all other quality control test results are acceptable. Remember that avirulent ND vaccines such as I-2 are safe in high doses, but this is not the case for other types of live ND vaccines.*

Example 2

If 5 mL allantoic fluid is harvested from each egg, how many eggs must be inoculated to produce 10 000 doses of vaccine? Assume that all eggs inoculated with I-2 ND working seed virus reacted positively to the HA test and are harvested.

Answer

1 mL = 40 drops (doses)

1 dose = $\frac{1.0 \text{ mL}}{40}$ = 0.025 mL

Volume of wet I-2 ND vaccine = 10 000 doses × 0.025 mL

= 250 mL

The vaccine is prepared by mixing 1 part of allantoic fluid with 1 part of 1% gelatin in PBS.

Volume of allantoic fluid in 250 mL wet I-2 ND vaccine = 0.5 × 250

= 125 mL

Number of eggs needed = $\frac{125 \text{ mL allantoic fluic}}{5 \text{ mL per egg}}$

= 25 eggs

To produce 10 000 doses of vaccine, 25 eggs must be inoculated. However, in practice, you will always incubate and inoculate more eggs to allow for non-specific embryonic death and inoculation errors.

3.7.2 Exercises

1. Allantoic fluid collected from eggs is diluted 1 to 1 with 1% gelatin in PBS to make wet I-2 ND vaccine. The titre of the vaccine is found to be 10^9 EID_{50} per 0.1 mL. The vaccine is stored at 22°C and samples are collected for titration after 2, 4, 6, 8 and 10 weeks in storage. The volume of one dose is 30 µL.

	Titre after storage					
	Day 0	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
EID_{50} per 0.1 mL	9	7.8	6.8	6.8	6.5	5.8
EID_{50} per mL						
EID_{50} per dose						

   (a) What is the titre of the 'wet' vaccine per mL at each time point? Per dose?

   (b) Is this vaccine suitable for use in the field (see Section 4.4)?

   (c) How many eggs must be inoculated to produce 25 000 doses of vaccine? Assume that all eggs inoculated with I-2 ND working seed virus reacted positively to the HA test and are harvested.

2. Allantoic fluid collected from eggs is diluted 1 to 1 with 4% skim milk in PBS to make wet I-2 ND vaccine. The titre of the vaccine is found to be $10^{8.8}$ EID_{50} per 0.1 mL. Plastic droppers that deliver 25 drops per mL are filled with the vaccine in the laboratory for delivery of the vaccine in the field. One dose is administered in one drop of vaccine. The vaccine is stored at 22°C and samples are collected for titration after 2, 4, 6, 8 and 10 weeks in storage.

	Titre after storage					
	Day 0	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
EID_{50} per mL	8.8	7.7	7.3	6.5	6.2	5.2
EID_{50} per dose						
EID_{50} per dose						

(a) What is the titre of the 'wet' vaccine per mL at each time point? Per dose?

(b) Is this vaccine suitable for use in the field (see Section 4.4)?

(c) How many eggs must be inoculated to produce 80 000 doses of vaccine? Assume that all eggs inoculated with I-2 ND working seed virus reacted positively to the HA test and are harvested.

The answers to the exercises are given in Appendix 11.

4.0

I-2 ND vaccine testing

4.1 Collecting blood from the wing vein of chickens

This technique is used to collect blood for the preparation of red blood cell suspensions (used in haemagglutination and haemagglutination inhibition tests) and serum for antibody testing. If the blood is for the preparation of red blood cell suspensions, the collection is made into an anticoagulant such as Alsever's solution or acid-citrate-dextrose (ACD) solution.

Equipment and materials

25 G (0.50 × 16 mm) needle for chicks under 4 weeks of age
or 23 G (0.65 × 32 mm) needle for older chickens
1.0 or 2.5 mL plastic syringes if collecting blood for serum
or 3 mL syringe (up to 10 mL may be used) if collecting blood for red blood cell suspensions.

Procedure

Before you begin, make sure that the needle is firmly attached to the syringe; that the needle cover is loose and easy to remove and that the plunger moves easily within the syringe.

With an assistant	Working alone
1. Ask the assistant to hold the chicken horizontally against themself with the chicken's head to their right.	1. Sit with the chicken held horizontally between your thighs, head away from you, lying half on its back and half turned on its right side. Hold down its legs with your left elbow (if you are right-handed) and its neck with your left forearm. With your left hand, spread out its left wing; with your right hand, take hold of the right wing (some people prefer to hold the birds with the head towards them; if you can learn to collect blood using both positions, you will have two veins to choose from).
2. Pull the right wing out towards you. If necessary pluck away the small feathers from the underside overlying the humerus, and swab with 70% alcohol. The wing vein, named in various textbooks as the brachial, ulnar or cutaneous ulnar, is clearly visible running between the biceps and triceps muscles.	
3. Insert the needle under the tendon of the pronator muscle, in the triangle formed where the wing vein bifurcates (Figure 8), pointing the needle proximally — that is, in the direction of the venous blood flow. Do not go too deep or the needle will scrape the humerus and the chicken will struggle. Keep clear of the ulnar nerve. With a little gentle probing you should enter the vein easily. This approach from under the tendon makes it easier to enter the vein than does aiming directly for it, and also tends to steady the needle if the bird moves.	
4. For serum collection, withdraw 0.5–1.0 mL of blood by gentle suction into a 2 mL syringe. Take care, because the veins of chickens collapse readily.	
5. After the needle is removed, apply pressure to the vein for a few seconds to discourage further bleeding.	

If the blood is required for preparation of a red blood cell suspension:
Collect the blood into a syringe containing anticoagulant. Mix the blood gently while it is in the syringe, remove the needle and transfer the blood to a vessel with a screw cap. If the blood is discharged through the needle, some of the red blood cells may lyse (rupture).

If the blood is required for serum:
Do not use an anticoagulant in the syringe. Immediately after collection of the blood, pull back the plunger of the syringe a little to leave an air space between the blood and the tip of the syringe. Label the syringe with the number of the chicken and date of collection and place the syringe on a flat surface or at a slight angle, with the needle end pointing upwards. This allows a larger surface area of blood to be exposed to air and will aid clotting.

Place the samples in a warm place and allow the serum to separate. Leave the blood at 37°C for at least four hours or at room temperature overnight to obtain good separation of the clot.

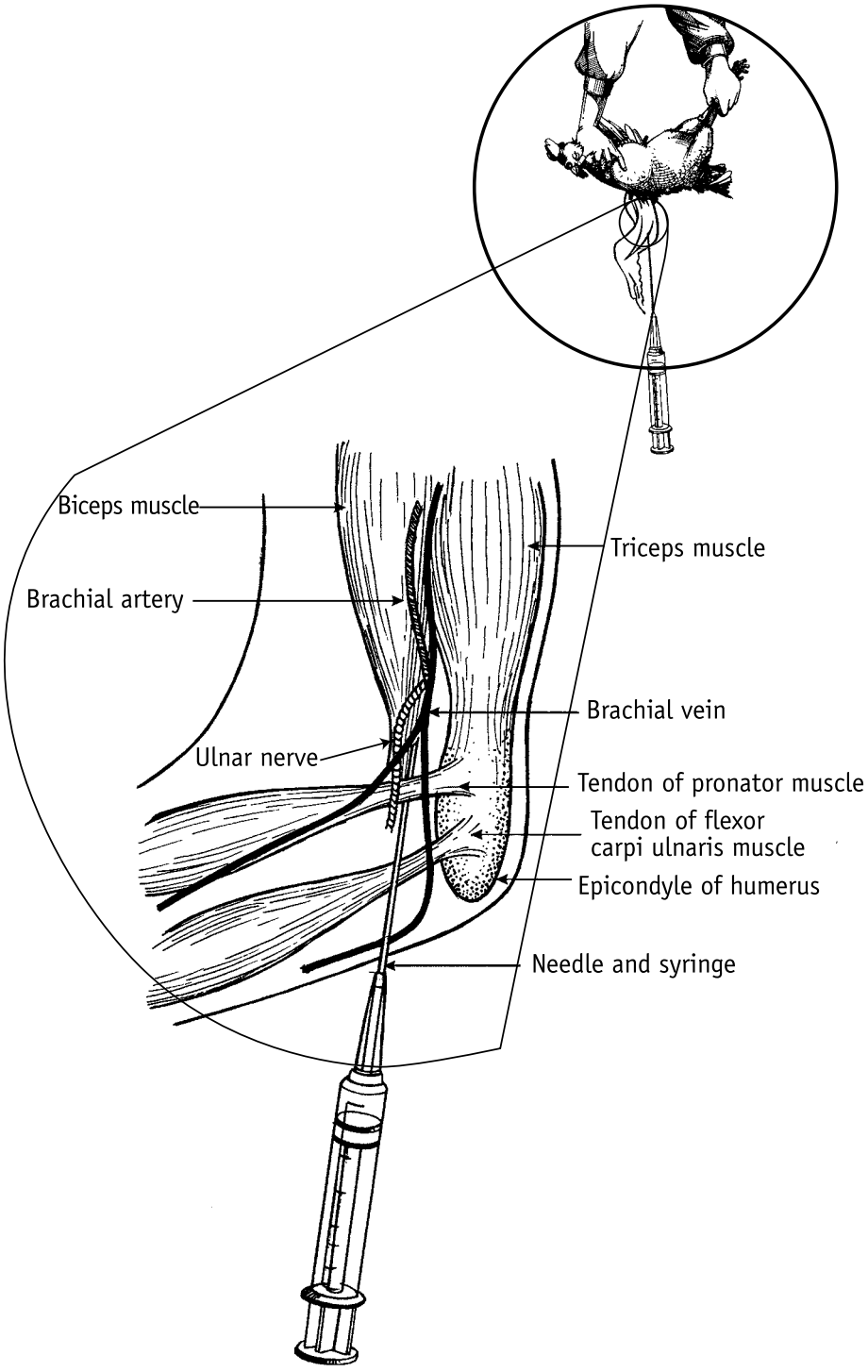


Figure 8: *Collecting blood from the brachial (wing) vein of a chicken.*

4.2 Preparing a washed red blood cell suspension

Washed red blood cell suspensions are used in haemagglutination (HA) and haemagglutination inhibition (HI) tests. Best results are obtained if the blood used to prepare the suspension is collected from a minimum of three ND virus antibody-free chickens and pooled (Thayer and Beard 1998). If this is not possible, blood from chickens vaccinated against ND virus or from chickens of unknown antibody status may be used as long as the red blood cell washing is thorough (Thayer and Beard 1998). It should be possible to collect a volume of blood equivalent to around 1% of the body weight from the wing vein of each adult donor chicken each week.

If vaccine production staff has access to a haematocrit centifuge and reader, it can be used to standardise the 10% red blood cell suspension. If a haematocrit centrifuge and reader are not available, it is possible to use graduated conical centrifuge tubes.

Blood volume of chickens

The blood volume of a chicken is around 7% of its body weight. A convenient rule of thumb is to collect a volume of blood equivalent to no more than 1% of the chicken's body weight (i.e. 1 mL from a 100 g chick) at each bleeding. Birds are more tolerant to blood loss than mammals since blood volume is replaced rapidly by resorption of tissue fluids.

Bounous and Stedman (2000)

Equipment and materials

Sterile glass screw-capped bottle (20 to 30 mL) or centrifuge tube
Anticoagulant — for example, Alsever's solution or ACD
Centrifuge
Red blood cell storage solution — for example DGV or PBS

Procedure

A. Using a calibrated centrifuge tube

1. Measure the required volume of anticoagulant into a sterile screw-capped bottle or centrifuge tube. Use one part of ACD to three parts of blood, or equal volumes of Alsever's solution and blood.
2. Draw the anticoagulant (Alsever's solution or ACD) into a 10 mL syringe.
3. Collect blood from one donor chicken (see Section 4.1). Gently mix the blood and anticoagulant in the syringe.
4. Remove the needle and discharge the blood into the bottle or centrifuge tube containing anticoagulant. Roll gently to mix the blood.
5. Collect blood from the remaining donor chickens (repeat steps 3 and 4 for each chicken).
6. Fill the bottle with DGV or PBS and mix gently.
7. Centrifuge at 500 × *g* for 10 minutes to sediment the red blood cells.
8. Remove the supernatant using a Pasteur pipette and discard. Do not disturb the red blood cell layer at the base.
9. Refill the bottle or centrifuge tube with DGV or PBS.
10. Mix gently to resuspend the cells and centrifuge again for 10 minutes at 500 × *g*. Rough mixing may cause haemolysis of the red cells.
11. Remove the supernatant using a Pasteur pipette and discard.

12. Refill the bottle or centrifuge tube with DGV or PBS.
13. Mix gently to resuspend the cells.
14. Pour the blood into 10 mL calibrated centrifuge tubes and centrifuge again for 10 minutes at 500 × *g*.
15. Remove the supernatant using a Pasteur pipette and discard.
16. Measure the volume of the red blood cell layer using the graduations on the wall of the centrifuge tube.
17. Add DGV or PBS to the red blood cell layer to make a final 10% red blood cell suspension. For example, if the volume of the red blood cell layer is 1 mL, add 9 mL DGV or PBS to make a total volume of 10 mL.
18. Mix gently to resuspend the cells, transfer the red blood cell suspension to a screw-capped bottle, label and store at 4°C.

B. Using a haematocrit centrifuge

Follow the procedure in *A. Using a calibrated centrifuge tube*, steps 1 to 11.

12. Add 10 mL DGV or PBS to the red blood cell layer and mix gently to resuspend the red blood cells.
13. Fill two microhaematocrit tubes with resuspended blood. Seal the dry end of the tubes with plasticine.
14. Place the microhaematocrit tubes in the haematocrit centrifuge with the sealed ends pointing to the outside of the centrifuge. Turn on the centrifuge and allow to spin for three minutes.
15. Remove the microhaematocrit tubes and read the packed cell volume (PCV) of the red blood cell suspension in a microhaematocrit reader.
16. Centrifuge the tube containing the red blood cell suspension again for 10 minutes at 500 × *g*.
17. Remove the supernatant using a Pasteur pipette and discard.
18. Add DGV or PBS so that the final concentration of red blood cells in the suspension is 10%. For example, if the PCV was 12%, add 12 mL of storage solution to the red blood cell layer. Label and store at 4°C.

NOTE:

- Handle the blood gently. Rough mixing or discharging of blood through a needle may cause some red blood cells to lyse.
- Do not use red blood cell suspensions that show signs of haemolysis (the clear fluid above the red blood cell is slightly red).

Storage solutions for red blood cell suspensions

Ten per cent red blood cell suspensions may be stored in DGV, Alsever's solution or PBS for several days at 4°C if no haemolysis is observed (Rovozzo and Burke 1973; Allan, Lancaster and Toth 1978; Thayer and Beard 1998). Trials at the John Francis Virology Laboratory, University of Queensland, showed that blood cells stored in DGV retained their integrity and function (measured by PCV and optical density, and HA test respectively) longer than red blood cells stored in either PBS or Alsever's solution. Red blood cell suspensions prepared from younger chickens (approximately 7 months old) were superior to suspensions prepared from older layer birds (approximately 14 months old).

4.3 Testing for the presence of virus (haemagglutination test)

All strains of ND virus and some other avian viruses and bacteria have compounds (haemagglutinins) on their envelopes that will attach to special sites (called receptors) on the surface of red blood cells, causing them to clump together (Thayer and Beard 1998). This process is called haemagglutination and is depicted in Figure 9. The clumps of red blood cells settle in specific patterns on glass slides or agglutination plates, or in the wells of plastic microtitre plates. In our experience, this test is best performed using plastic 96-well V-bottomed microtitre plates (see Section 4.3.2).

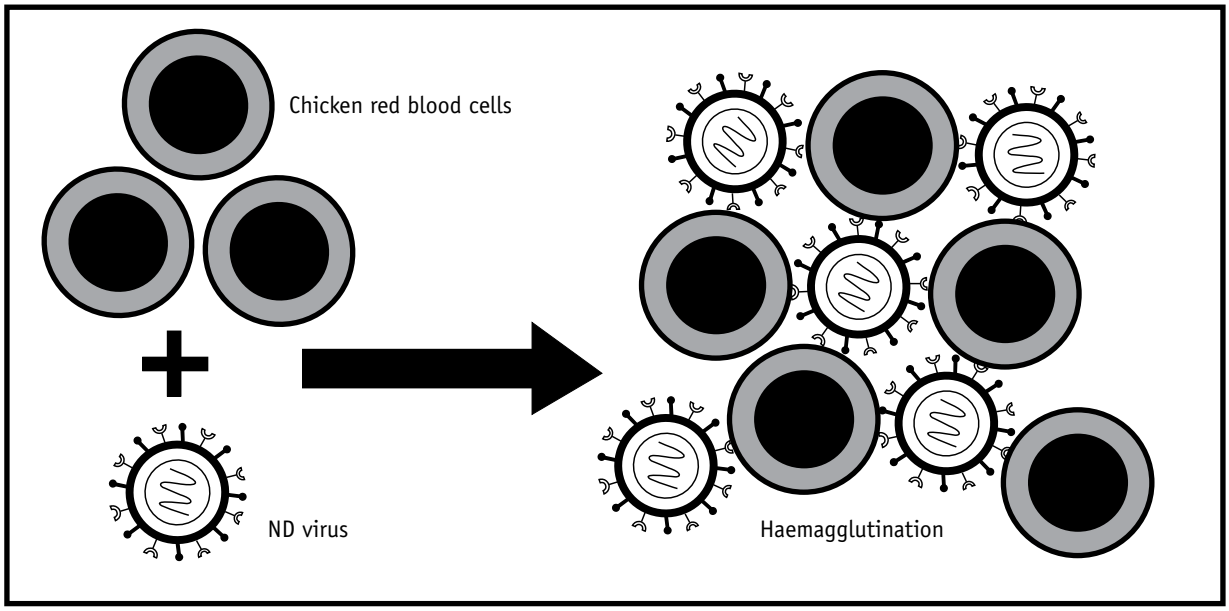


Figure 9: *The principle of haemagglutination.*

The haemagglutination (HA) test is used to determine the presence of viral haemagglutinin in a sample. It can also be used to measure the quantity of haemagglutinin present. Therefore, the HA test is used to confirm the presence of ND virus in the allantoic fluid of eggs inoculated with I-2 ND virus for vaccine production or for titration of a sample, to indicate the amount of virus in the vaccine and to standardise the amount of haemagglutinin used as antigen in the haemagglutination inhibition (HI) test.

NOTE:

- The HA test gives an indication of the 'amount' of virus in the sample, but does not tell if the virus is viable (live). To determine if the virus is viable, the infectivity of the virus must be measured by titration in embryonating eggs.
- Other avian viruses, including influenza virus and egg drop syndrome virus as well as several *Mycoplasma* spp., are also able to agglutinate red blood cells. To prove that the agent causing haemagglutination is ND virus, it is necessary to inhibit the haemagglutinating activity with specific ND positive serum (Thayer and Beard 1998).

4.3.1 Rapid haemagglutination test

Equipment and materials

10% red blood cell suspension
PBS
Sterile glass Pasteur pipettes
Agglutination plates, microscope slides or white tiles

Procedure

1. Withdraw one drop of allantoic fluid from each egg through a small hole made in the shell membrane. Use a separate sterile glass pipette for each egg.
2. Place the allantoic fluid in wells on an agglutination plate or on tiles or microscope slides. Place drops in sequence to correspond to the position of the eggs on the tray.
3. Add 1 drop of fresh 10% red blood cell suspension to each drop of allantoic fluid and mix gently using a swirling motion.
4. After 1 minute, check the wells for agglutination. The clumps of agglutinated cells will be seen easily.
5. Record the results.

Positive: clumping of red blood cells — indicates the presence of haemagglutinin (+)

Negative: no clumping of red blood cells — indicates the absence of haemagglutinin (–)

4.3.2 Haemagglutination test

Equipment and materials

10% red blood cell suspension
PBS
Pipettor and sterile tips
96-well V-bottomed microtitre plates

Procedure

1. Prepare a 1% suspension of red blood cells from the 10% suspension by taking one part (1 mL) of the suspension and mixing with nine parts (9 mL) of PBS.
2. Using a pipettor and sterile plastic tips, withdraw 50 µL of allantoic fluid from each egg through a small hole made in the membrane or using the hole made during injection. Use a separate sterile plastic tip for each egg. Place each drop in a separate well of the microtitre plate.
3. Add one drop of 1% red blood cell suspension (25 µL) to each drop of allantoic fluid. Mix gently.
4. After 45 minutes, examine and record the settling pattern.

Positive: a thin film of red blood cells — indicates the presence of haemagglutinin (+)

Negative: a sharp button of red blood cells — indicates the absence of haemagglutinin (–)

When red blood cells settle in the wells of a microtitre plate, single (non-agglutinated) cells will settle and roll to the lowest part of the well, forming a small, well-defined button. Clumps of cells (agglutinated) will settle in the wells but remain where they settle. The bottom of the well will be covered with a thin film of red blood cells. This is shown in Figure 10.

NOTE:

- For titration of a virus suspension, the HA test using V-bottomed microtitre plates is recommended. Although the test must be allowed to stand for 45 minutes, the results are easy for a beginner to read. In addition, the test is sparing of red blood cell suspension and plates can be disinfected, washed and reused several times.
- Always include positive and negative controls in the haemagglutination tests.

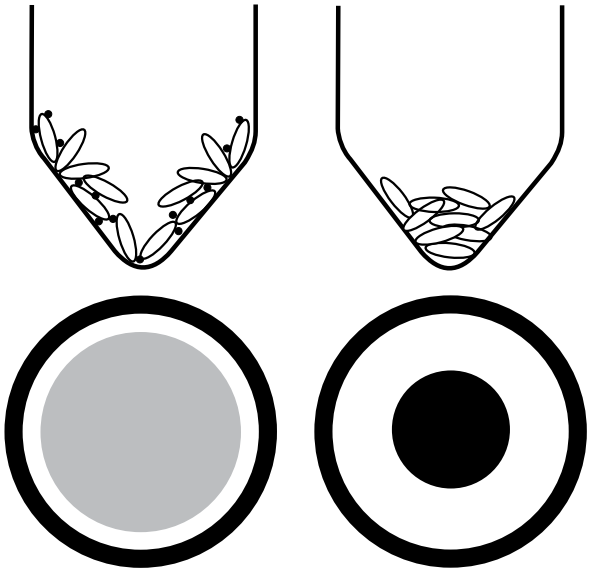


Figure 10: *The settling pattern of agglutinated and non-agglutinated red blood cells in V-bottomed microtitre plates.*

4.3.3 Titration of haemagglutinin using the quantitative haemagglutination test

The quantitative haemagglutination test measures the amount of virus in a sample (for example, vaccine or allantoic fluid) by titration. In a titration the strength of the suspension of virus is judged from the extent to which it can be diluted before it fails to show signs of virus activity (in this test, haemagglutination). The quantitative haemagglutination test is also used to measure and standardise the amount of haemagglutinin used as the antigen in the HI test. Twofold serial dilutions of the sample are made (see Appendix 8) and the number of haemagglutinating units in the sample is measured. The steps of this procedure are shown in Figure 11.

Equipment and materials

10% red blood cell suspension
PBS
Pipettes
Multichannel pipettor and tips (to measure 25 µL)
96-well plastic microtitre plates with V-bottomed wells

Procedure

1. Prepare a 1% suspension of red blood cells from the 10% suspension by taking one part (1 mL) of the suspension and mixing with nine parts (9 mL) of PBS.
2. Using the multichannel pipettor, dispense 25 µL of PBS into each well of two (sample in duplicate) or three (sample in triplicate) rows of the microtitre plate.
3. Place 25 µL of virus suspension (for example, allantoic fluid) into the first well of each row (this is a 1:2 dilution).
4. Mix well and make twofold dilutions of the suspension across the row by transferring 25 µL of fluid from one well to the next. Discard 25 µL from the last well of each row so that the volumes in each well will be the same (see Appendix 8).
5. Dispense 25 µL PBS into each well of a control row. This row will show the normal settling patterns and time of red blood cells in suspension.
6. Add 25 µL PBS to each well (including the control wells).

7. Add 25 µL of 1% red blood cell suspension to each well.
8. Mix gently and allow to stand at room temperature for 45 minutes. Cover the plate to stop dehydration.
9. Read the results and record.

Positive: a thin film of red blood cells — indicates the presence of haemagglutinin (+)

Negative: a sharp button of red blood cells — indicates the absence of haemagglutinin (–)

The last well that shows complete haemagglutination is said to contain one haemagglutinating (HA) unit. From this, the titre of the original undiluted sample may be calculated. For example, if the sixth well (1:64) is the last to show haemagglutination, then the original material contained 64 (2^6) HA units.

One HA unit is defined as the highest dilution of antigen which will completely agglutinate a test dose of red blood cells under standard conditions of temperature and time of incubation.

NOTE:

- Red blood cell control wells should always be included in the test. These wells contain 50 µL PBS and 25 µL 1% red blood cells, but no virus. At the end of the reaction time, these wells should show no haemagglutination — that is, there should be a button of red blood cells at the base of the well. The wells show the normal settling patterns and time of red blood cells in suspension.
- It is important that the test results are read as soon as possible after the 45 minute incubation period has ended. After some time, the virus elutes — that is, the agglutinated cells disassociate (separate) and red blood cells will roll to the base of the wells. Thus, a positive sample may not show haemagglutination and could appear as a negative sample. Remember that different strains of ND virus elute at different rates and elution of the virus occurs more rapidly at higher temperatures.

4.4 Estimating the concentration of live ND virus

Although the HA test measures the ability of a virus suspension to agglutinate red blood cells, it does not give any information about whether the virus is live, inactive or in the form of viral subunits. Therefore, it is not a reliable estimate of the amount of viable virus in a sample. To confirm the presence and measure the amount of live ND virus in a suspension, a virus infectivity assay (performed by the inoculation of eggs or tissue culture) is used. If the suspension is a sample of I-2 ND vaccine, the infectivity titration is a measure of vaccine potency.

The infectivity of a suspension of ND virus is measured by preparing serial dilutions (usually tenfold) of the virus suspension, inoculating these into eggs, incubating the eggs and determining the highest dilution at which the virus can still be detected in the allantoic fluid, using the haemagglutination test.

The unit of infectivity used is the mean (50%) embryo infective dose (EID_{50}) — that is, the amount of virus capable of infecting 50% of inoculated eggs. For strains of ND virus that kill embryos, the mean embryo lethal dose (ELD_{50}) can be calculated. This is the titre of the virus suspension and is expressed as the number of infectious units per unit volume (for example, per mL of solution or per dose of vaccine).

The end point of the titration is the highest dilution of virus that will produce a detectable effect (in this case, haemagglutination) in 50% of inoculated eggs. This is determined mathematically by the method of Reed and Muench (Villegas 1998).

Haemagglutination test

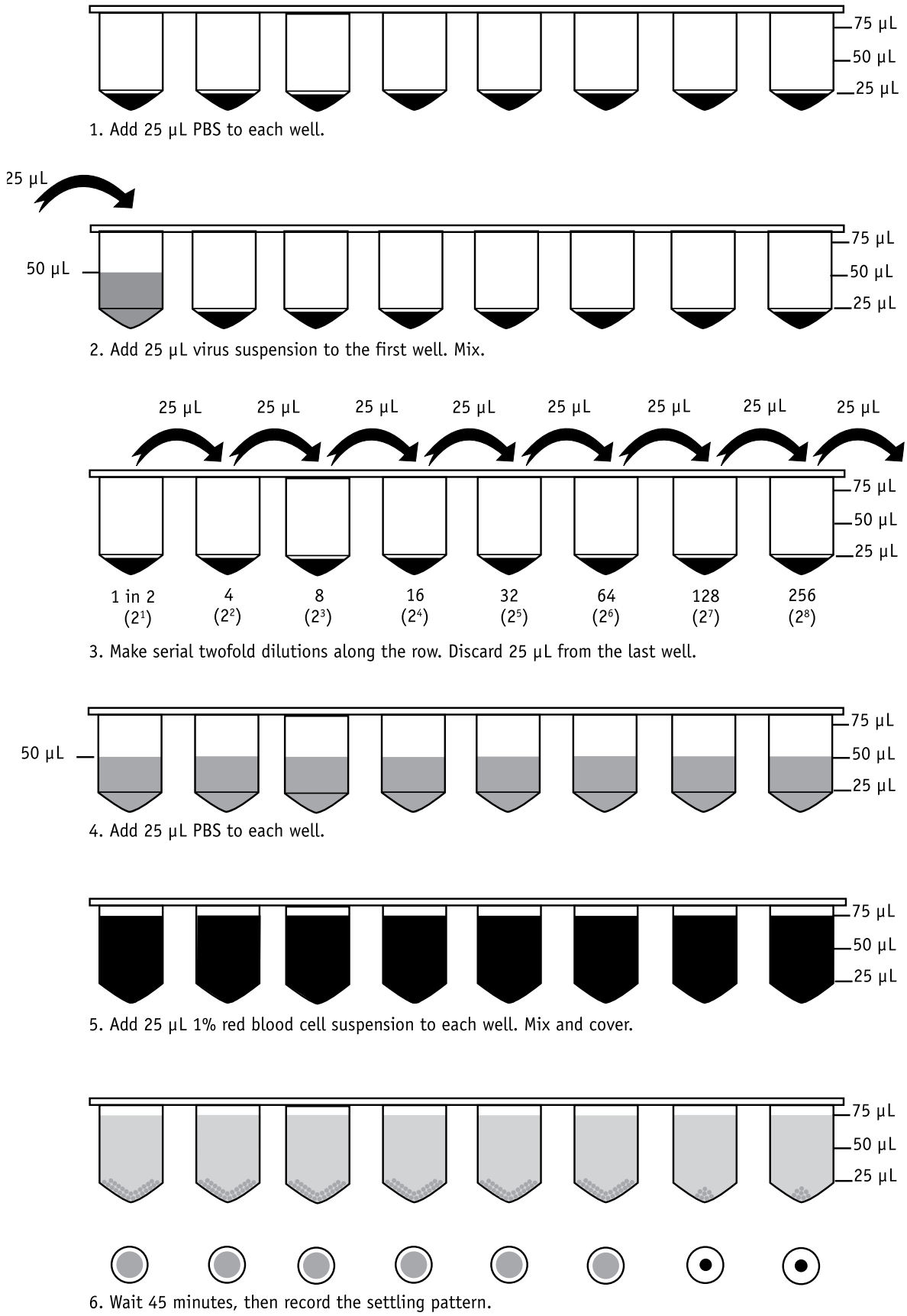


Figure 11: *The haemagglutination test is performed by combining a washed red blood cell suspension and ND virus. The HN glycoprotein on the ND virus attaches to receptors on the red blood cells, causing agglutination.*

Equipment and materials

PBS
Sterile glass tubes
Glass pipettes (1 and 10 mL) or pipettors (200–1000 µL and 10–200 µL) and tips
Syringe
Needles
Egg shell disinfectant — for example, 70% alcohol or iodine in alcohol
Colloidin, adhesive tape or paraffin/petroleum jelly mixture to seal the inoculation site

Procedure

Inoculations must be performed using aseptic technique.

1. Label sterile glass tubes in series from the lowest dilution of virus suspension to be tested to the highest (for example, 10^{-1}, 10^{-2} to 10^{-10}).
2. Pipette 0.9 mL of PBS into each tube.
3. Prepare tenfold serial dilutions of the virus suspension, using a clean sterile pipette or pipette tip to transfer 0.1 mL of virus suspension from one tube to the next each time (see Appendix 8).
4. Select five fertile eggs for each dilution. Prepare the eggs for inoculation (candle and mark the inoculation site). Write the date of inoculation and dilution of the virus suspension on the shell of each egg in pencil. If several titrations are being done at the one time it is also necessary to identify each tray with a batch number, diagnostic number or technician's initials.
5. Disinfect the inoculation site and punch a hole at the marked site.
6. Inoculate the eggs. The same syringe and needle can be used to inoculate all eggs in the titration if the highest dilution of virus suspension (least concentrated: for example 10^{-10}) is inoculated first.
7. Seal the inoculation site. Incubate the eggs for 4 days, candling at 24 and 72 hours. Record any deaths that occur during the incubation period. Discard eggs containing dead embryos if measuring the infectivity of I-2 ND vaccine (or other avirulent strains). If titrating virulent strains, perform a rapid HA test on eggs containing dead embryos.
8. After four days of incubation, cool the eggs at 4°C for 4 hours.
9. Swab the shells with disinfectant and open the eggs.
10. Collect a drop of allantoic fluid from each egg and perform the haemagglutination test (see Section 4.3.1 or 4.3.2).
11. Record the results. An example with 10 rows each containing five eggs is given below.

Dilution	Results				
10^{-1}	+	+	+	+	+
10^{-2}	+	+	+	+	+
10^{-3}	+	+	+	+	+
10^{-4}	+	+	+	+	+
10^{-5}	+	+	+	+	+
10^{-6}	+	+	+	+	–
10^{-7}	+	+	+	–	–
10^{-8}	+	+	–	–	–
10^{-9}	–	–	–	–	–
10^{-10}	–	–	–	–	–

Positive: a thin film of red blood cells — indicates the presence of haemagglutinin (+)

Negative: a sharp button of red blood cells — indicates the absence of haemagglutinin (–)

NOTE:

- The diluent used must be non-toxic and preserve the infectivity of the virus. PBS is ideal. Antibiotics may be added to the PBS used to dilute the virus suspension.
- Separate sterile pipettes or pipette tips must be used to prepare and mix each dilution. The used pipette will carry virus particles on its outer surface, and if it is not discarded after each transfer, the particles will be carried along from one dilution to the next. Dilutions will be inaccurate and after inoculation into eggs and incubation for 96 hours, these virus particles will multiply, resulting in a large error.
- Deaths up to 24 hours post-inoculation are considered non-specific and are not included in the calculations.
- I-2 ND virus is harmless for embryos for at least 148 hours post-inoculation via the allantoic cavity. If deaths have occurred after 24 hours, the cause of the deaths should be investigated (see Section 5.10).
- The titration should include the full range of results — that is, from 100% infected to 0% infected. If this has not occurred, the titration should be repeated. However, if eggs are scarce, the values either side of the 50% endpoint can be calculated and if the results are consistent, then it is possible to calculate the titre using the existing results.

Calculating the titre

The titre of the virus suspension can be calculated according to the mathematical technique devised by Reed and Muench (1938).

Procedure

1. Write the numbers of infected (HA positive) and uninfected (HA negative) eggs in the table below.

Dilution	Number of infected eggs	Number of uninfected eggs	Accumulated total infected eggs	Accumulated total uninfected eggs	Ratio and % infected eggs (accumulated)
10^{-2}			↑	↓	
10^{-3}			↑	↓	
10^{-4}			↑	↓	
10^{-5}			↑	↓	
10^{-6}			↑	↓	
10^{-7}			↑	↓	
10^{-8}			↑	↓	
10^{-9}			↑	↓	
10^{-10}			↑	↓	

The number of infected eggs is generally 0 to 5 per dilution (total may be less than 5 if embryos have died).

The number of uninfected eggs is generally 0 to 5 per dilution (total may be less than 5 if embryos have died).

2. 'Accumulate' the results for infected eggs in the direction of the arrows by adding each number (or result of the previous addition) to the next, beginning from 0 infected (see worked example in Section 4.4.1).
3. 'Accumulate' the results for uninfected eggs in the direction of the arrows by adding each number (or result of the previous addition) to the next, beginning from 0 uninfected (see worked example in Section 4.4.1).
4. Calculate the ratio and percentage of infected eggs at each dilution using the following equations:

$$\text{Ratio} = \frac{\text{accumulated total of infected eggs}}{(\text{accumulated total infected eggs} + \text{accumulated total uninfected eggs})}$$

$$\text{Percentage} = \text{ratio} \times 100$$

5. Look at the percentage of infected eggs and locate the values closest to 50%: one above and one below 50%. Note the dilutions that correspond to these values. The dilution that produced the 50% endpoint lies between these dilutions.
6. To calculate the exact dilution that produced infection in 50% of inoculated eggs, use the Reed and Muench formula to calculate the index (also called proportionate distance in some textbooks).

$$\text{Index} = \frac{(\%\text{ infected eggs at dilution immediately above 50\%}) - 50\%}{(\%\text{ infected eggs at dilution above 50\%}) - (\%\text{ infected eggs at dilution below 50\%})}$$

7. The index shows how close the dilution that produced infection in 50% of inoculated eggs is to the dilution that produced the percentage of infected eggs immediately above 50%.
8. Calculate the 50% endpoint using the formula:

$$\text{Log of 50\% endpoint} = (\text{log dilution above 50\%}) - (\text{index} \times \text{log dilution factor}^{a})$$

[a] Where tenfold dilutions have been used, the log dilution factor is 1.0.

9. The titre is the reciprocal (positive value) of the dilution that produced 50% infection (the negative exponential of the endpoint dilution). Since the volume of the inoculum was 0.1 mL, the titre is expressed as EID_{50} per 0.1 mL.

Some workers prefer to use the method of Spearman-Kärber (Villegas 1998) to calculate the titre of virus. Generally, this method does not involve a great number of calculations, and the results are reported to be as accurate as those obtained using the Reed and Muench formula. However, this method can only be used where the results of the dilutions tested cover the full 0–100% infection range. Worked examples using this method are shown in Appendix 7.

Minimum titres

The minimum dose of I-2 vaccine required to provoke a protective immune response in most chickens is 10^6 EID_{50} (Alders and Spradbrow 2001). However, to ensure the minimum dose is present at the time a chicken is vaccinated in the field, it is best to produce vaccine with not less than 10^7 EID_{50} per dose. This will allow for any losses in titre that may occur during storage, transport and field use (Alexander 1991). Therefore, for I-2 ND vaccine:

The **minimum production titre** is 10^7 EID_{50} per dose (this is also called the laboratory standard dose).

The **minimum field titre** is 10^6 EID_{50} per dose.

4.4.1 A worked example

One vial of freeze-dried I-2 vaccine is reconstituted with 2.5 mL sterile PBS. The volume of vaccine in the vial before freeze-drying was 2.5 mL, sufficient for 250 doses.

A series of dilutions of this vaccine is made from 10^{-1} to 10^{-10} and 0.1 mL of each dilution is inoculated into the allantoic cavity of five 9- to 11-day-old embryonating eggs. At the end of 96 hours incubation, a sample of allantoic fluid from each egg is tested for the presence of viral haemagglutinin by the haemagglutination test. The results are shown in the table below.

1. Calculate the infectivity titre of the vaccine. Express the titre as EID_{50} per 0.1 mL, per 1.0 mL and per dose.
2. The minimum production titre of vaccine required by the laboratory is 10^7 EID_{50} per dose. Is the titre of this vaccine acceptable?

Answer 1

Dilution	HA results	No. of infected eggs	No. of uninfected eggs	Accumulated total infected eggs	Accumulated total uninfected eggs	Ratio and % accumulated infected eggs
10^{-1}	+ + + + +	5	0	34	0	
10^{-2}	+ + + + +	5	0	29	0	
10^{-3}	+ + + + +	5	0	24	0	
10^{-4}	+ + + + +	5	0	19	0	
10^{-5}	+ + + + +	5	0	14	0	(14/14) 100
10^{-6}	+ + + + –	4	1	9	1	(9/10) 90
10^{-7}	+ + + – –	3	2	5	3	(5/8) 62.5*
10^{-8}	+ + – – –	2	3	2	6	(2/8) 25
10^{-9}	– – – – –	0	5	0	11	(0/11) 0
10^{-10}	– – – – –	0	5	0	16	

+ Infected.
– Uninfected.

Using the method of Reed and Muench:

1. Fill in the number of infected eggs and the number of uninfected eggs (columns 3 and 4).
2. Calculate the accumulated totals of infected and uninfected eggs (columns 5 and 6), as shown.
3. Calculate the ratio of infected to uninfected eggs, and the percentages (column 7). The percentage shown with * should be rounded off (approximated) to 63.

It can be seen from this table that the 50% endpoint is between 10^{-7} (62.5%) and 10^{-8} (25%).

4. Use the following formula to calculate the index (or proportionate distance) between 10^{-7} and 10^{-8}.

$$\text{Index} = \frac{(\%\text{ infected eggs at dilution immediately above 50\%}) - 50\%}{(\%\text{ infected eggs at dilution above 50\%}) - (\%\text{ infected eggs at dilution below 50\%})}$$

$$\text{Index} = \frac{63 - 50}{63 - 25} = \frac{13}{38} = 0.34$$

The number 0.34 should be rounded off to 0.3.

5. Calculate the 50% endpoint using the formula:

Log of 50% endpoint = (log dilution above 50%) – (index × log dilution factor[a])
= –7 – (0.3 × 1.0) = –7.3

Therefore, the dilution that produced 50% infection = $10^{-7.3}$.

[a] Since tenfold dilutions have been used, the log dilution factor is 1.0.

6. Calculate the titre of the vaccine (the number of infectious units per unit volume).

Per volume of inoculum:
The dilution that produced 50% infection = $10^{-7.3}$. The titre is the reciprocal (positive value) of the dilution that produced 50% infection.

Since the volume of the inoculum was 0.1 mL, each 0.1 mL contains $10^{7.3}$ EID_{50}.

Per mL:
(1 mL = 10^{1} × 0.1 mL)

Each 1 mL contains $10^{7.3} \times 10^{1}$ EID_{50} = $10^{8.3}$ EID_{50}

Per dose:
The vial contains sufficient virus for 250 doses and was reconstituted in 2.5 mL sterile PBS.

$$\text{Volume of one dose} = \frac{2.5 \text{ mL}}{250 \text{ doses}} = 0.01 \text{ mL}$$

$$\begin{aligned}\text{Titre per dose} &= 10^{8.3}\ EID_{50} \text{ per mL} \times 0.01 \text{ mL} \\ &= 10^{8.3}\ EID_{50} \text{ per mL} \times 10^{-2.0} \text{ mL} \\ &= 10^{8.3-2.0}\ EID_{50} \text{ per mL} \\ &= 10^{6.3}\ EID_{50} \text{ per dose}\end{aligned}$$

Answer 2

The titre of this vaccine is less than the minimum production titre. If this is freshly made vaccine and records show that production protocols were followed, the test should be repeated. If the same result is obtained, the batch of vaccine should be rejected. If this is from a batch of vaccine that has been stored for some time, review the production and storage records and the titre at production.

Rounding off

When calculating the results of a titration, it is usual to 'round off' or approximate the decimals. This will simplify the mathematical operations and have little effect on the accuracy of the results.

- When rounding off percentages, any figure with a decimal of 0.5 or greater should be rounded up to the nearest whole number and any figure with a decimal less than 0.5 should be rounded down.
- When rounding off the index, any figure with a decimal of 0.05 or greater should be rounded up to the nearest single decimal place and any figure with a decimal less than 0.05 should be rounded down. For example, 0.16 would be rounded up to 0.2 and 0.14 would be rounded down to 0.1.

4.4.2 Exercises

A series of dilutions of vaccine is made from 10^{-1} to 10^{-10}; 0.1 mL of each dilution is inoculated into the allantoic cavity of five 9- to 11-day-old embryonating eggs. At the end of 96 hours incubation, a sample of allantoic fluid from each egg is tested for the presence of viral haemagglutinin by the haemagglutination test. The results are shown in the tables below (these exercises are examples only and do not reflect the real results of titrations).

The symbols +, – and 0 represent the results of the haemagglutination test, that is:

+ Infected
– Uninfected
0 Non-specific embryonic death during incubation

Calculate the titre of the vaccine (per 0.1 mL, per 1.0 mL and per 30 µL dose).

The minimum production titre of vaccine required by the laboratory is 10^7 EID_{50} per dose. Is the titre of this vaccine acceptable?

The answers to the exercises are given in Appendix 11.

Exercise 1

Dilution	HA results	No. of infected eggs	No. of uninfected eggs	Accumulated total infected eggs	Accumulated total uninfected eggs	Ratio and % accumulated infected eggs
10^{-1}	+ + + + +					
10^{-2}	+ + + + +					
10^{-3}	+ + + + +					
10^{-4}	+ + + + +					
10^{-5}	+ + + + +					
10^{-6}	+ + + + +					
10^{-7}	+ + + – –					
10^{-8}	+ + – – –					
10^{-9}	+ – – – –					
10^{-10}	– – – – –					

Exercise 2

Dilution	HA results	No. of infected eggs	No. of uninfected eggs	Accumulated total infected eggs	Accumulated total uninfected eggs	Ratio and % accumulated infected eggs
10^{-1}	+ + + + +					
10^{-2}	+ + + + +					
10^{-3}	+ + + + +					
10^{-4}	+ + + + +					
10^{-5}	+ + + + +					
10^{-6}	+ + + + +					
10^{-7}	+ + + + +					
10^{-8}	+ + + – –					
10^{-9}	+ – – – –					
10^{-10}	– – – – –					

Exercise 3

Dilution	HA results	No. of infected eggs	No. of uninfected eggs	Accumulated total infected eggs	Accumulated total uninfected eggs	Ratio and % accumulated infected eggs
10^{-1}	+ + + + +					
10^{-2}	+ + + + +					
10^{-3}	+ + + + +					
10^{-4}	+ + + + +					
10^{-5}	+ + + + +					
10^{-6}	+ + + + –					
10^{-7}	+ + + – –					
10^{-8}	+ + – – –					
10^{-9}	+ – – – –					
10^{-10}	– – – – –					

Exercise 4

Dilution	HA results	No. of infected eggs	No. of uninfected eggs	Accumulated total infected eggs	Accumulated total uninfected eggs	Ratio and % accumulated infected eggs
10^{-1}	+ + + + +					
10^{-2}	+ + + + +					
10^{-3}	+ + + + +					
10^{-4}	+ + + + +					
10^{-5}	+ + + + +					
10^{-6}	+ + – – –					
10^{-7}	+ + – – –					
10^{-8}	+ – – – –					
10^{-9}	– – – – –					
10^{-10}	– – – – –					

Exercise 5

Dilution	HA results	No. of infected eggs	No. of uninfected eggs	Accumulated total infected eggs	Accumulated total uninfected eggs	Ratio and % accumulated infected eggs
10^{-1}	+ + + + +					
10^{-2}	+ + + + 0					
10^{-3}	+ + + 0 0					
10^{-4}	+ + + + –					
10^{-5}	+ + + 0 0					
10^{-6}	+ + – – 0					
10^{-7}	+ + – – 0					
10^{-8}	+ – – – –					
10^{-9}	– – – – –					
10^{-10}	– – – – –					

4.5 Testing vaccine for the presence of contaminants

4.5.1 Testing vaccine for freedom from bacterial and fungal contaminants

Each batch of I-2 ND vaccine should be tested and confirmed free of viable bacterial and fungal contaminants that could be harmful to chickens receiving the vaccine. National registration authorities should provide vaccine producers or distributors with details of the tests required. Suitable tests are also outlined in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (2011) and the *Manual of ASEAN standards for animal vaccines* (1998) together with information on interpretation of the test results. The procedures for testing live viral vaccines administered through drinking water, by spray or skin scarification are used

(OIE 2011e) since specific guidelines for vaccines administered by eye-drop are not given.

In general, two test methods are recommended for detection of bacterial and fungal contaminants: the membrane filtration test and the direct inoculation sterility test. For most laboratories producing I-2 ND vaccine, the direct inoculation sterility test will be the method of choice. In this test, a sterile pipette or syringe and needle are used to transfer a sample of the vaccine directly into liquid media. Liquid media are chosen since they provide a better environment than solid media for detecting small numbers of viable organisms present in a sample. It is also possible to 'quantify' the number of organisms in a sample and dilute inhibitory substances, for example antibiotics, when using liquid media.

The media used should support the growth of a wide range of micro-organisms. Fluid thioglycollate medium (FTM) and soyabean-casein digest medium (SCDM, also known as trypticase soy medium) are recommended. FTM is used mainly for culture of anaerobic bacteria, but will also detect aerobic bacteria. SCDM is intended for the culture of aerobic bacteria, but is also suitable for fungi. All media used should be tested for their sterility and ability to support the growth of micro-organisms before they are used to test for vaccine contaminants.

Detection of contamination in a batch of vaccine with absolute certainty would involve the examination of every vial of vaccine using a range of media capable of supporting the growth of all possible contaminants. Clearly this is impossible. Therefore, a protocol for sampling should be established that ensures that representative samples of the entire batch are tested and that sufficient samples are collected to detect contaminants with acceptable levels of probability.

In the testing of I-2 ND vaccine for bacterial and fungal contaminants, a two-stage testing protocol is recommended. Samples of pooled allantoic fluid from each collecting vessel and a representative number of the filled vaccine containers — for example, glass vaccine vials — should be collected and tested. The number of containers of vaccine to be tested in each batch is determined by the number of containers produced in the batch and is based on the probability of detecting one contaminated container in a batch of that size.

- If the batch size is not more than 100 then 10% or four containers, whichever is the greater, should be tested.
- If the batch contains between 100 and 500 containers then 10 containers should be tested.
- If the batch has more than 500 containers then 2% or 20 containers, whichever is the lesser, should be tested.

It is also important to consider the volume of each container.

- If the volume of vaccine in each container is less than 1 mL, then 0.5 mL is inoculated into each medium.
- If the volume in each container is from 1 to 4 mL, then half of the contents are used in each medium.
- If the volume is from 4 to 20 mL, then 2 mL of the contents is inoculated into each medium.

If micro-organisms are present in the sample, the medium may appear turbid or a sediment may form and the batch of vaccine should be retested. If no microbial growth is detected in any of the vessels inoculated with the vaccine, then the batch complies with the tests for freedom from contamination. For vaccines administered through drinking water, by spray or skin scarification, 'a limited number of contaminating, non-pathogenic bacteria and fungi may be permitted'.

For poultry vaccines, each final container of the vaccine should contain not more than one bacterial or fungal colony per dose (OIE 2011e).

4.5.2 Testing vaccine for freedom from extraneous viral agents

Some small laboratories may have difficulty testing vaccine for freedom from extraneous viral agents. If the vaccine is destined for use in village chickens, then the cheapest option is to monitor the layer flock that produce the eggs for vaccine production to minimise the risk that vertically transmitted viral diseases are present. This is discussed further in Section 3.3.

Vaccine destined for use in commercial flocks is beyond the scope of this manual. Appropriate protocols for testing of vaccine for freedom from extraneous viral agents are available.

NOTE:

The effect of any vaccine contaminant on the chicken will be affected by the:

- type of contaminant
- degree of contamination
- route of administration of the vaccine
- the resistance or susceptibility of the individual bird.

4.6 Laboratory trials of I-2 ND vaccine

I-2 ND vaccine has undergone laboratory tests in several countries and has proved to be protective against local virulent strains of ND virus (Amakye-Anim et al. 2000; Tu et al. 1998; Hlaing et al. 2000; Wambura, Kapaga and Hyera 2000). National vaccine producers or distributors should undertake vaccine trials in their own country in order to provide evidence of the potency and efficacy of the vaccine in accordance with the requirements of regulatory authorities. In addition, the producer may wish to:

- compare the levels of protection afforded by different routes of administration of the vaccine and different administration regimes
- confirm that the I-2 ND vaccine strain provides protection against local virulent strains of ND virus, and
- train laboratory and field staff.

> All veterinary biological products administered to animals should be tested for safety, and if possible, for efficacy in the field using good clinical practice, before being authorised for general use. Field studies are designed to demonstrate efficacy under working conditions and to detect unexpected reactions, including mortality that may not have been observed during the development of the product. Under field conditions there are many uncontrollable variables that make it difficult to obtain good efficacy data, but demonstration of safety is more reliable.
>
> The tests should be done on the host animal, at a variety of geographical locations, using appropriate numbers of susceptible animals. The test animals should represent all the ages and husbandry practices for which the product is indicated; unvaccinated controls must be included. The product tested should be one or more production batches/serials. A protocol should be developed indicating the observation methods and the recording methods.
>
> OIE (2011c)

In addition to fulfilling regulatory requirements to confirm that the vaccine is safe and potent under field conditions, field trials provide an excellent opportunity to design, test and evaluate extension material that is adapted to local conditions.

A detailed description of the design, materials and methods and protocol of laboratory and field trials is given in Alders and Spradbrow (2001). Table 4 shows a suggested chronology for laboratory trials. SPF chickens are best, but if these are not available then it is possible to use chickens from a commercial flock. The chickens should be collected at day old (or hatched under control conditions in the laboratory) and kept in isolation until maternal antibodies have waned (that is, at approximately three weeks of age).

Table 4 *Chronology for laboratory trials*

Day 1	Place chicks in an isolated brooder.
Day 21 (3 weeks)	1. Allocate chicks randomly to experimental groups (minimum 10 birds per group), including a control group. 2. Place groups in an experimental chicken unit with the control (unvaccinated) group in a separate isolation unit attended by different animal attendants, if possible. 3. Wing tag chicks and collect a serum sample from each. 4. Vaccinate the test groups, recording batch number and expiry date of vaccine. 5. Include unvaccinated 'in contact' chicks in each group (to test for the spread of the virus between birds). 6. Perform HI test on all serum samples.
Day 35 (5 weeks)	1. Collect serum samples from all birds. 2. Perform HI test on all serum samples.
Day 49 (7 weeks)	1. Collect serum samples from all birds. 2. *If chickens are to be challenged with virulent ND virus, place chickens inoculated intranasally with the virulent challenge strain in contact with vaccinated and control groups. Observe all groups daily. Record clinical signs and deaths.* 3. Perform HI test on all serum samples.
Day 63 (9 weeks)	1. Collect serum from all surviving chickens. 2. Perform HI test on all serum samples.

NOTE:

- Fully susceptible unvaccinated 3-week-old chicks must be used (HI titre less than 2^1), at least 10 birds per group. If you are unsure of the antibody status of the chicks, collect serum and perform HI test on all samples *before* birds are allocated to groups. Only chicks with HI titre less than 2^1 should be included in the trial.
- Since pathogenic forms of NDV are used in challenge studies, the facility used to house the birds should meet the OIE requirements for Containment Group 4 pathogens (OIE 2011d).
- Control birds should be kept in a separate isolation unit and strict biosecurity implemented to prevent the spread of virus between groups.
- The route of vaccination should correspond to the usual route of field application.
- The route of challenge should simulate challenge in the field where the natural route of infection is by inhalation or ingestion of infectious virus (Alexander 1998). Using these routes of infection, the protection provided by circulating, cellular and mucosal immunity is tested. Although it is not possible to know accurately the challenge dose per bird using these routes, if a bird dies then it is certain that the challenge dose contains at least one mean chicken lethal dose (MCLD).

Although intramuscular injection provides the most reproducible lethal effect in fully susceptible birds and ensures that the challenge dose is known, this is an unnatural route of infection. In addition, only the protection afforded by circulating or humoral antibody is tested.

- Potent vaccines of the lentogenic type should provide full protection following one field dose administered by eye-drop.

4.7 Preparing serum

Equipment and materials

2 mL Eppendorf tubes or cryotubes with a screw cap and flat base
Rack or storage container

Procedure

1. Collect a sample of 0.5 to 1.0 mL of blood from the wing vein of the chicken. Label the syringe with the number of the chicken.
2. Pull back the plunger of the syringe to leave an air space between the blood and the end of the syringe. Ensure that there is no blood in the tip of the syringe. Place the syringe at an angle with the needle end pointing upwards (to increase the surface area for clotting).
3. Place the samples in a warm place and allow the serum to separate. Good separation of the clot is obtained if the blood is left at 37°C for at least four hours or at room temperature overnight.
4. Carefully remove the needle from the syringe and hold the tip of the syringe over an Eppendorf tube. Remove the plunger from the syringe. The clot should remain attached to the plunger and the serum should flow from the tip of the syringe into the tube.
5. If the serum does not flow from the syringe easily or if the clot does not remain attached to the plunger, it may be necessary to pour the serum into the Eppendorf tube or to use a glass Pasteur pipette to remove the serum. Remove the label from the syringe and place it on the Eppendorf tube.
6. Centrifuge the Eppendorf tubes in a microcentrifuge for 30 seconds and pour the serum into new Eppendorf tubes or cryotubes, taking care not to disturb the red blood cell pellet. Remove the label from the used Eppendorf tube and place it on the new Eppendorf tube or cryotube.
7. Place the tubes in a rack or storage container to keep them upright.
8. For short-term storage (up to 2 weeks), place the serum at 4°C. For long-term storage, place the serum in a freezer at –20°C or –70°C.

NOTE:

- Handle the blood gently. Haemolysed or contaminated serum samples may give false test results.
- Store the tubes upright during freezing. This will ensure that the serum remains at the base of the tube and will reduce any losses that may occur when tubes are opened after thawing.
- Storage of serum at –70°C is ideal since enzymes in serum will continue to degrade proteins stored at –20°C (Catty and Raykundalia 1988).
- It is best to divide each serum sample into two aliquots if many tests are to be carried out on the one sample. Repeated cycles of freezing and thawing may affect the test results.

- Suitable labels for syringes and tubes can be made from Dermicel tape or masking tape. Such labels can be transferred from the syringe to the Eppendorf tube and then to the final serum storage tubes. This saves time and reduces mistakes in transcribing numbers and dates.

4.8 Testing for antibody (haemagglutination inhibition test)

Chickens infected with ND virus through natural field exposure or vaccination will develop antibodies to the virus. Antibodies can usually be detected and measured in the serum six to ten days after infection with ND virus using a number of serological tests. The haemagglutination inhibition (HI) test is the test most commonly used for detecting antibodies to ND virus. It is easy to perform, economical and expensive equipment is not required.

The basis of the HI test is the inhibition (prevention) of haemagglutination by specific antibodies. If ND virus is added to serum containing antibodies to ND virus, the antibody will bind to the virus. ND virus bound to antibody will not be able to react with red blood cells and haemagglutination will not occur. Where there is more virus than antibody, haemagglutination will be observed since there will still be some free virus available. Where there is more antibody than virus or sufficient antibody to react with all the virus, haemagglutination will not be observed.

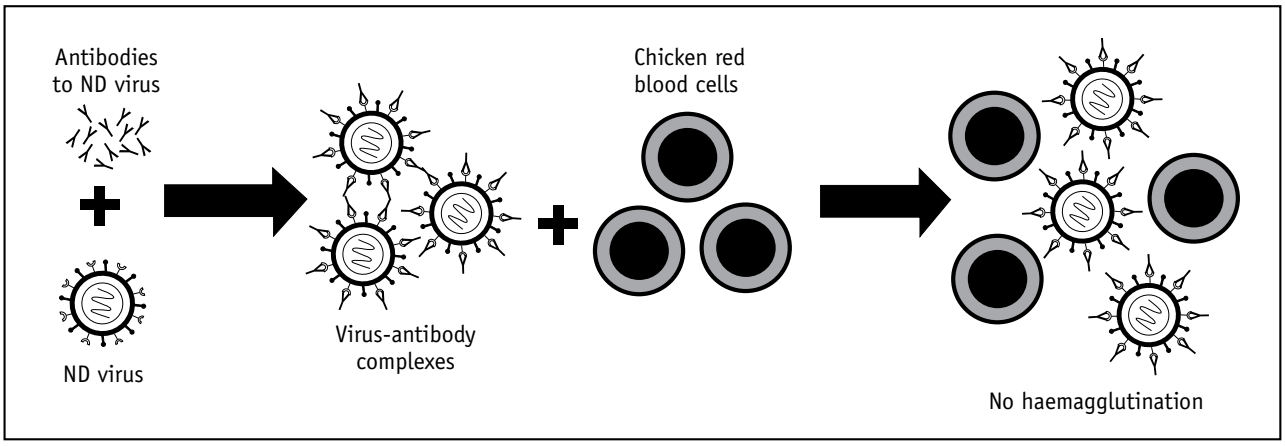


Figure 12: *The principle of haemagglutination inhibition.*

There are a number of variations in the way the test is performed and these will influence the final test results. For example, the concentration of HA antigen (ND virus) used will affect the sensitivity of the test and serum antibody titres will vary with the amount of antigen used. An increased concentration of HA antigen in the test (to 8 HA units) results in decreased sensitivity while a decreased concentration results in increased sensitivity. Other variables may also affect the results: for example, the concentration of red blood cell suspension; the time between the mixing of the serum and the antigen and the addition of the red blood cells; and the temperature and the criteria used for reading the test (Thayer and Beard 1998). Therefore, it is important that a standard protocol be used for the test since this will enable comparison of the results within and between laboratories.

The technique described here uses 4 HA units (Allan and Gough 1974) and gives the most consistent results in our laboratories. The test is performed in V-bottomed plates since the settling pattern of the red blood cells is easier to read.

4.8.1 Preparing antigen for the haemagglutination inhibition test

The antigen used in the HI test is allantoic fluid containing ND virus. This may be prepared from a batch of I-2 ND vaccine produced in the vaccine production unit or a vial of vaccine prepared from an avirulent strain of ND virus, such as Ulster or V4 (Maas et al. 1998). Avirulent strains of ND virus give more reliable results in the HI test. If a trial requiring a large number of HI tests is planned, prepare a large batch of antigen and use the same batch throughout the trial. Store the antigen in 1 mL aliquots at –20°C.

4.8.2 Haemagglutination inhibition test

Each day before the HI test is performed on the test serum samples, a number of pre-test checks must be performed.

- The number of HA units in the antigen suspension must be determined using the HA test. The antigen suspension is then diluted so that 25 µL contains 4 HA units.
- A 'back titration' is performed on the diluted suspension to confirm that the dilution is correct.
- The final pre-test check confirms that the positive and negative control sera are giving the expected results in the HI test.

If these pre-test checks are not done, valuable serum samples may be wasted and results of the HI test may be unreliable and inaccurate.

Equipment and materials

PBS
ND virus antigen suspension
1% red blood cell suspension
96-well V-bottomed microtitre plates
Glass pipettes (1 and 10 mL)
Pipettors (200–1000 µL and 10–200 µL) and tips
Negative control serum (see Section 4.8.4)
Positive control serum (see Section 4.8.5)
Test sera

Procedure

Preparation of 4 HA units of ND virus antigen suspension

1. Using the quantitative HA test (see Section 4.3.2), titrate the ND virus antigen suspension and calculate the HA titre.
2. Divide the HA titre by four to calculate the dilution factor.
3. Calculate the volume of diluted antigen suspension required. Allow 2.5 mL for each microtitre plate.
4. Measure the volume of antigen suspension required and dilute in PBS, using the dilution factor calculated above.

A worked example

The HA titre of the ND virus antigen suspension is 1:64 (2^6).

1. Work out the dilution factor.
2. Calculate the volume of diluted antigen suspension needed for four plates.
3. Calculate the volume of antigen suspension and the volume of PBS that must be mixed to produce this volume of diluted antigen suspension.

1. Dilution factor = $\frac{2^6}{4} = \frac{64}{4} = 16$

Therefore, the ND virus antigen suspension must be diluted 1:16 before it is used in the HI test (1:16 dilution means that one part of antigen suspension must be mixed with 15 parts of PBS to give a final concentration of 4 HA units).

2. If four plates are to be used:

The volume of 4 HA units required = 4 plates × 2.5 mL/plate = 10 mL

It is prudent to prepare a little more antigen than for your exact, immediate needs. Additional antigen may be required to replace spillage or to repeat doubtful tests. This also allows the laboratory worker to select a volume that allows simple mathematical calculations, thus reducing the chance of error. In the present example, the required volume of working antigen is 10 mL. The allantoic fluid (64 HA units in 25 µL) must be diluted 1 in 16 to produce the working antigen (4 HA units in 25 µL). A convenient method would be to dilute 1 mL of allantoic fluid in 15 mL of PBS, yielding 16 mL of working antigen at the required strength. Other solutions would be possible, for example 0.8 mL of allantoic fluid in 12.0 mL of PBS.

Calculations are easily made for greater volumes of working antigen or for different dilution factors. Always remember, no matter how certain you are of your mathematics, include a titration of the working antigen in your test.

Back titration of diluted antigen suspension

5. Using the HA test (see Section 4.3.2), titrate the diluted ND virus antigen suspension.
6. Confirm that the HA titre of the diluted suspension is 4 (that is, 2^2).

Confirmation of titre of standard positive and negative sera

7. Using the HI test (see procedure below), titrate the positive and negative sera.
8. Confirm that the HA titre of the positive serum is 2^5 (see Section 4.8.5) and of the negative serum 2^1 or less (see Section 4.8.4).

Test Sera

9. Calculate the number of plates required and number each plate with a marking pen. If there is sufficient serum, it is good to do the tests in duplicate.
10. Add 25 µL PBS to each well of a 96-well V-bottomed microtitre plate.
11. Shake serum gently to mix. Add 25 µL of serum to the first and last (control) well of each row.
12. Using a multichannel pipettor, make serial twofold dilutions of each serum sample along the row by transferring 25 µL of fluid from one well to the next (see Appendix 8). Stop at the second last well.

13. Discard 25 µL of fluid from the second last well of the row. Do not dilute the last (control, twelfth) well of each row.
14. Add 25 µL of 4 HA units to each well (not to the control well).
15. Tap the side of the plate gently to mix, cover and allow to stand at room temperature for 30 minutes.
16. Add 25 µL of a 1% suspension of red blood cells to each well.
17. Tap the side of the plate gently to mix, cover and allow to stand at room temperature for 45 minutes.
18. Read the agglutination pattern. Record results as shown in Figures 13 and 14: no haemagglutination ⦿ and haemagglutination ◉.
19. Determine the endpoint. This is the well that shows complete inhibition of haemagglutination.
20. Record the antibody titre for each sample. The titre is recorded as the highest dilution of serum that causes complete inhibition of haemagglutination. HI results from individual birds are usually expressed as the reciprocal (positive value) of the end point serum dilution.

NOTE:

- **Controls should always be included in the HI test:**
  - — wells containing positive and negative control sera (with known HI titre)
  - — a well containing serum, PBS and red blood cell suspension only, but no antigen. This is included as a control for red blood cell suspension and tests for natural agglutinins in serum.
- It is important that the test results are read as soon as possible after the 45 minute incubation period has ended. After some time, the virus elutes — that is, the agglutinated cells disassociate and red blood cells will roll to the base of wells. Thus, a negative serum sample may not show haemagglutination and could appear as a positive serum sample. Elution of the virus occurs more rapidly at higher temperatures.
- Read the control wells first.
- The volume of serum available for testing from each chicken is often quite small. Therefore, it is important that the number of HA units in the antigen is standardised and the titres of control negative and positive sera are confirmed before this valuable test serum is used.
- If the serum sample in the last well of the row (twelfth well) shows haemagglutination, natural agglutinins are present in the sample and will need to be absorbed (see Section 4.8.6).
- Chicken sera rarely contain non-specific inhibitors, but if present these will result in erroneous HI results. Samples may be heat inactivated in a water bath at 56°C for 30 minutes. If further inactivation is required, samples should be treated with potassium periodate (Burleson, Chambers and Wiedbrauk 1992).
- The endpoint is not always easy to determine. Look at the size of the red blood cell button and compare it with the size of the button in the control well.
- To conserve resources always consider the origin of the samples and the expected results before beginning the HI test.
- Vaccinated chickens that have survived challenge with virulent ND virus may develop titres of HI antibody greater than 2^{12} and the end point will not be reached in a single row of the plate.

Possible problems that may be encountered on the control plate

Back titration of 4 HA units:

- If haemagglutination occurs in more than the first two wells (1 and 2), then there are more than 4 HA units in the haemagglutinin. If a large quantity of the haemagglutinin solution was prepared, it should be titrated again and more PBS added to achieve 4 HA units. Alternatively, the haemagglutination titre of the original solution should be tested again and diluted accordingly to give 4 HA units.
- If haemagglutination occurs in fewer than two wells, then there are fewer than 4 HA units in the haemagglutinin. The haemagglutination titre of the original solution should be tested again and diluted accordingly to give 4 HA units.

Positive and negative control sera:

- If there are more than 4 HA units in the ND virus antigen suspension (>4 HA units means too much virus), the test will give a generally low HI titre or false negative HI results. The positive control will read lower than 32 (2^5).
- If there are fewer than 4 HA units in the ND virus antigen suspension (<4 HA units means too little virus), the test will give a generally high HI titre or false positive HI results. The positive control will read higher than 32 (2^5) and the negative control might read higher than 2^1.

To correct this problem, recalculate the dilution of the antigen to get 4 HA units and retest the control plate.

Red blood cell suspension:

- If the red blood cell suspension is not fresh and has haemolysed, cells might not agglutinate or settle down properly as buttons. It will be hard to read the endpoint. To correct the problem, use fresh red blood cell suspension.

A positive HI result shows past exposure to ND virus by vaccination or natural infection. To confirm recent infection, it is necessary to collect and test acute and convalescent sera from the same bird and confirm a rise in titre.

There is a good correlation between the level of ND-virus-specific antibodies and the degree of immunity in birds older than 6 weeks. The HI test allows the likely outcome of infection with virulent NDV at different levels of immunity to be predicted:

HI titre negative — <1:8 (2^3) — birds are probably not immunised or infected

HI titre positive — 1:8 (2^3) or higher — this level should protect against death in uncomplicated infections; will not prevent some replication of virulent virus; and egg production losses may occur.

Haemagglutination inhibition test

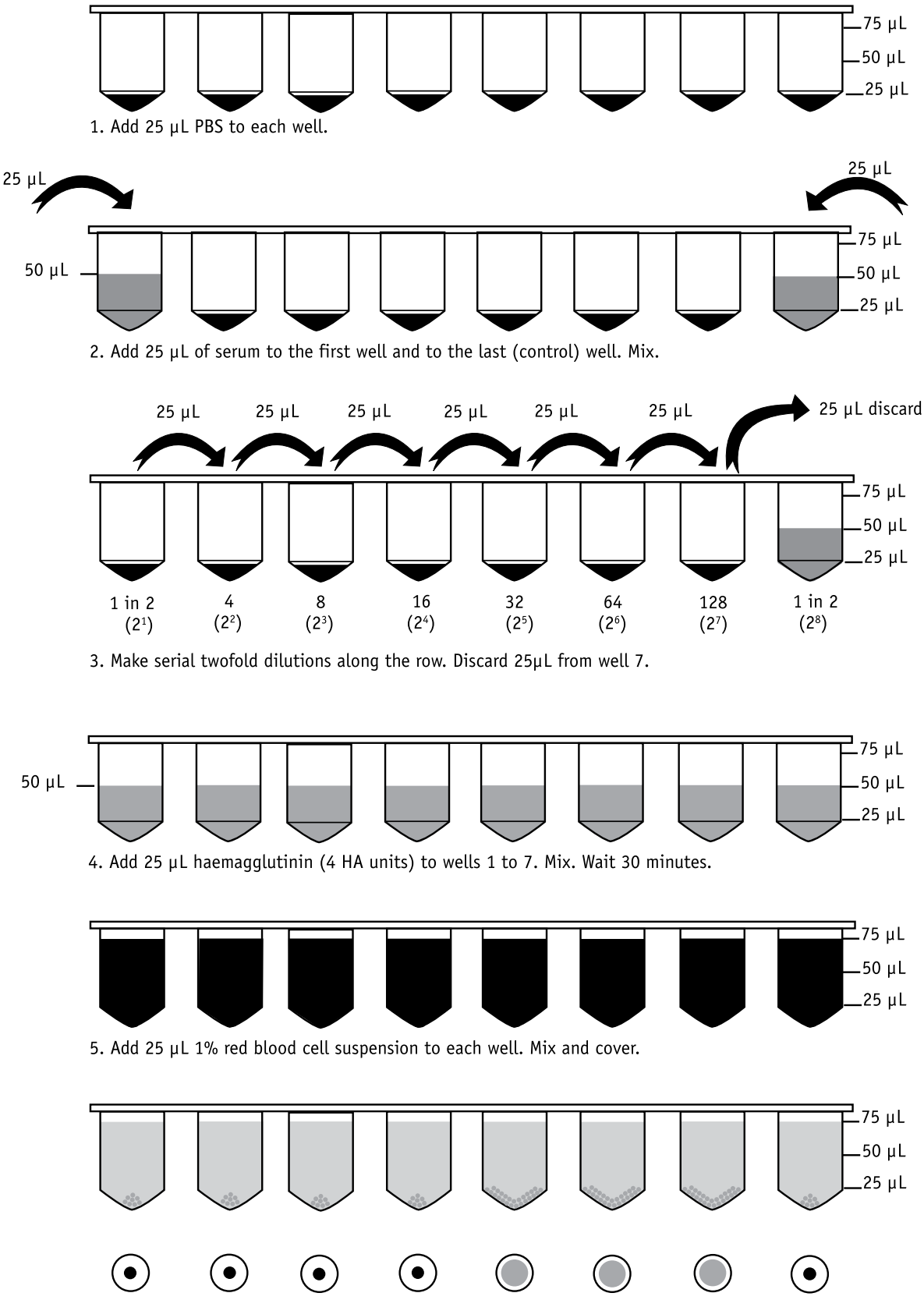


Figure 13: *The haemagglutination inhibition test.*

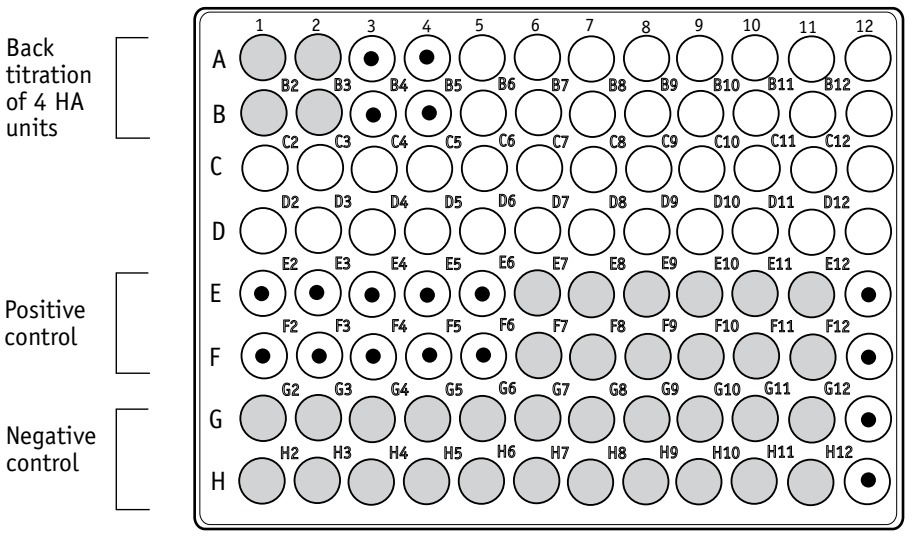


Figure 14: *A 96-well V-bottomed microtitre plate set up for the HI test. All titrations are done in duplicate. Rows A and B show the result of the back titration of 4 HA units (HA titre of 4 or 2^2). Rows E and F show the results of the titration of the positive control serum (HI titre of 32 or 2^5) and rows G and H, the results of the titration of the negative control serum (HI titre of 0 or 2^0 or no titre). Column 12 rows E to H show the normal settling pattern of non-agglutinated red blood cells. This well contains serum, PBS and red blood cell suspension only.*

4.8.3 Exercises

The four plates shown in Figure 15 show the results of HI tests performed on serum samples collected in the field by the District Veterinary Officer. The tests were performed in duplicate. Look at the plates and answer the following questions. Give explanations for your answers.

1. Did the HI test work as you expected?
2. What is the HI titre for each of the serum samples?
3. Will the chicken be protected if an outbreak of ND occurs?
4. Serum 1 was collected from a recently vaccinated bird. What should the titre have been? Why is the result so low?
5. Rows E and F of the first plate show the results of testing of sample 3 in duplicate. What could have happened to cause this inconsistency?
6. The titre of sample 8 (rows G and H of the second plate) is very high and cannot be determined on this test. If this is a correct reading, what could have caused it? How would you determine the actual titre of this sample? If you think that this high result is a technical error, what could have caused it?
7. Serum samples 9, 10, 13 and 14; tests were performed in duplicate but the results of the two tests are not the same. What would you do to confirm the results? If there is insufficient serum remaining to retest, what titre would be recorded on the report form?

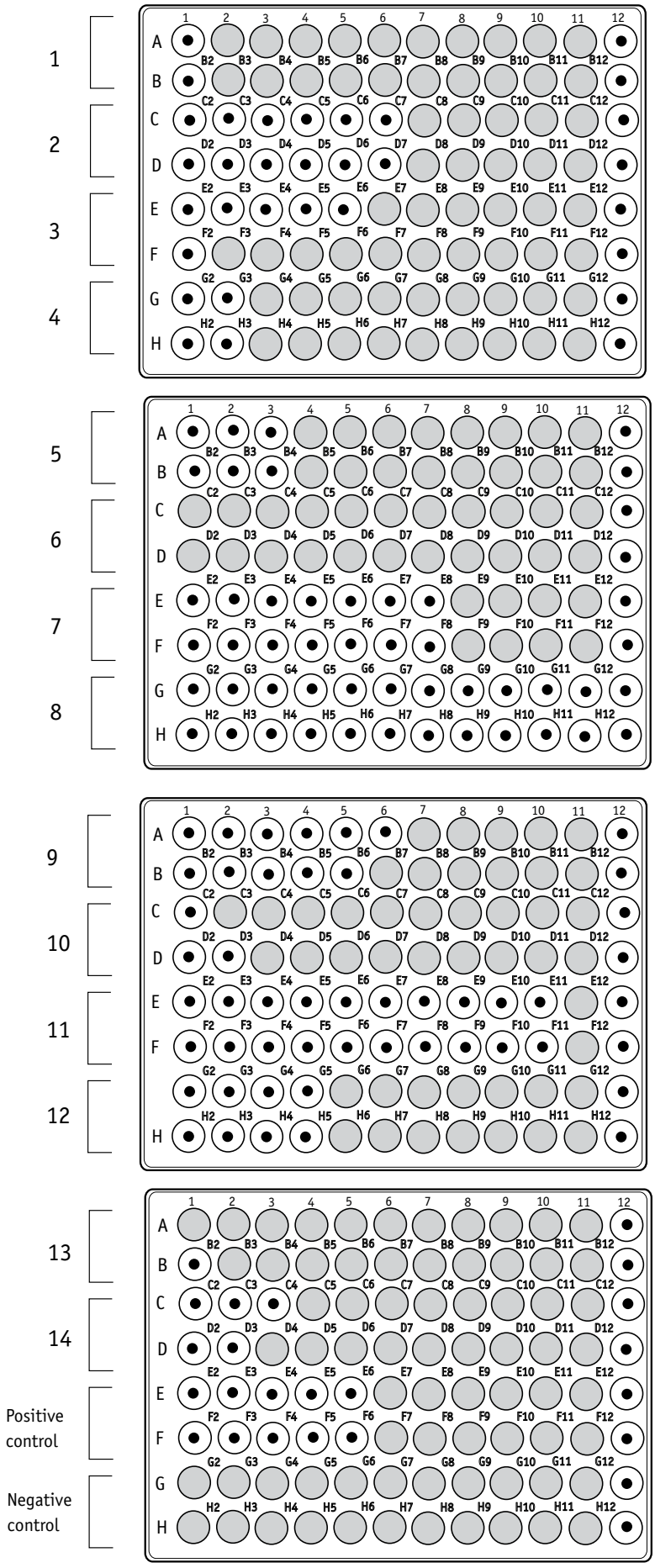


Figure 15: *HI test exercises.*

4.8.4 Preparing HI-negative control serum

The ideal HI-negative control serum contains no antibodies to the ND virus. It has no HI titre and does not agglutinate chicken red blood cells. If it is difficult to find chickens with serum with no HI titre, serum with low positive HI titres of 2^1 or 2^2 may be used.

Procedure

1. Collect blood from normal healthy chickens.
2. Allow the blood to clot.
3. Separate the serum from the clot, pool samples and centrifuge (see Section 4.7).
4. Test the serum using the HI test (see Section 4.8.2) to confirm that the serum shows a titre of 2^1 or less.
5. Store in 1 or 2 mL aliquots at –20°C.

NOTE:

In areas where it may be difficult to find birds with no or low HI titres, serum samples from individual birds should be tested and only those with suitable HI titres should be pooled.

4.8.5 Preparing HI-positive control serum

The HI positive control serum contains antibodies to the ND virus. The titre of the serum should be within the range 2^4 to 2^8. Reference serum is usually prepared by international or central national laboratories for distribution to smaller laboratories and is generally expensive to purchase. The national laboratory will then prepare a large volume of positive serum of matching titre for 'in-house' use or distribution to provincial or branch laboratories. The HI titre of the serum is established by repeated titration.

The following protocol is based on that described by Alexander (1998).

Procedure

1. Vaccinate several 6- to 9-week-old chickens with I-2 ND vaccine via eye-drop or nasal-drop.
2. Vaccinate the chickens again two weeks later.
3. Kill the chickens three weeks after the second vaccination and collect as much blood as possible.
4. Allow the blood to clot.
5. Separate the serum from the clot and pool the serum samples.
6. Centrifuge the serum and store in 1 or 2 mL aliquots at –20°C.
7. Test the serum by HI against reference positive serum to determine the HI titre. Use freshly titrated and diluted antigen. The titre of the serum should be within the range 2^4 to 2^8 (it is best to use serum with a titre of 2^4 or 2^5 since less serum will be required to produce the laboratory standard serum to match national or international standards).
8. Repeat several times to confirm the HI titre.

An additional test may be used to confirm the HI titre of the serum.

1. Prepare a series of twofold dilutions of the serum from 1:2 to 1:64.
2. Test by HI. The test results should show that the HI titre of the reference serum is equal to the predetermined HI titre (see above: 2^4 to 2^8).
3. Divide serum into aliquots, label and store in the freezer at –70°C. If –70°C is not available, –20°C is adequate.

Standard positive serum can also be prepared from stored HI-positive serum samples.

1. Pool serum samples with a titre equal to or greater than the reference positive control and test by HI against the reference positive control serum up to ten times. Use freshly prepared 4 HA units of antigen for each test. Test on different days if possible.
2. Compare the results. The most frequently occurring titre can be considered the HI titre of the standard serum.
3. Dilute in PBS or HI-negative serum until the titre of the pooled sera is equal to the titre of the reference positive control serum.

4.8.6 Adsorption of natural agglutinins

Sometimes the serum control wells in the HI test for particular chicken serum samples will show haemagglutination. These sera contain natural agglutinins that interact with red blood cells, causing agglutination. If the endpoint is obscured, the agglutinins must be removed by adsorption with chicken red blood cells so that the samples can be retested.

Equipment and materials

10% red blood cell suspension
Eppendorf tubes
Micropippetor (to 1000 µL) and tips
Microcentrifuge

Procedure

1. Place 200 µL 10% red blood cell suspension in an Eppendorf tube.
2. Centrifuge in the microcentrifuge for 15 seconds.
3. Remove the supernatant and discard.
4. Mix the serum sample by gentle shaking. Take 500 µL of the serum and add to the red blood cells in the Eppendorf tube. Mix gently.
5. Allow to stand for at least 30 minutes at 4°C.
6. Centrifuge in the microcentrifuge for 15 seconds.
7. Transfer the supernatant (serum) to a clean Eppendorf tube as quickly as possible.

NOTE:

Follow the instructions in steps 4 to 7 closely to minimise the risk of dissociation of natural agglutinins and red cells.

4.8.7 How to calculate the geometric mean titre (GMT)

The mean values of data such as HI titres that are expressed as logs are calculated and expressed as geometric means rather than arithmetic means. In this way the effects of results that are much larger or smaller than most are reduced.

The general formula for calculating the GMT is:

$GMT = \sqrt[n]{x_1 x_2 x_3 \ldots\ldots\ldots\ldots x_n}$

where x = value of the observation, n = number of observations.

In our experience, the following method gives good results.

To calculate the GMT:

1. Calculate the $\log_2$ of each of the titres in the group.
2. Add the indexes and divide by the total number of samples (where the HI titre is $<2^1$ the index is '0').
3. This is the geometric mean titre.

A worked example

The HI titres of four chickens vaccinated during a laboratory trial of I-2 ND vaccine were 2^3, 2^4, 2^4 and 2^6. Calculate the GMT.

$2^3 = 8$

$2^4 = 16$

$2^4 = 16$

$2^6 = 64$

Use Y^x function key on calculator

$$\text{GMT} = \frac{3 + 4 + 4 + 6}{4} = \frac{17}{4} = 2^{4.2} = 18.4$$

This would normally be written as GMT ($\log_2$) or HI titre ($\log_2$) = 4.2

(The arithmetic mean of these titres would be 26 due to the single high value, 2^6 i.e. $\frac{8 + 16 + 16 + 64}{4} = 26$)

4.9 Serological surveys

Serological surveys are an important tool in livestock disease control activities. They may be undertaken in an area to determine the prevalence of a disease such as ND and to assist in the planning, implementation and monitoring of disease control programs. Proper planning will ensure that scarce resources are not wasted and that reliable information is gathered quickly and cost effectively.

4.9.1 Estimating disease prevalence

The examples below are based on random sampling — that is, samples are collected at random from the population. Often this is not possible in field studies and some sort of cluster sample (birds only from particular owners, etc.) is collected. This complicates the estimation of disease prevalence. There are complicated formulae for estimating prevalence using cluster samples that are often impractical to use in the field situation. Often a compromise may need to be made with the use of random sampling formulae and an acceptance of a loss of precision in the estimate.

1. Sampling from a large (theoretically) infinite population using simple random sampling

The approximate sample size required to estimate disease prevalence can be determined using a set of specific tables — for example, Cannon and Roe (1982) which lists expected prevalence and desired precision at several confidence levels.

Alternatively, a simple formula may be used to give an approximation:

$$n = \frac{4 \times p \times (1 - p)}{L \times L}$$

where n = sample size required, p = expected prevalence, L = desired precision.

Unless the expected prevalence is known, it is best to use an expected prevalence of 50%.

Thus, the sample size to determine the disease prevalence in a large (infinite) population with an accuracy of +/–2% is:

$$n = \frac{(4 \times 50 \times 50)}{(2 \times 2)} = 2500$$

If the accuracy is reduced to +/–5%, then the sample size is:

$$n = \frac{(4 \times 50 \times 50)}{(5 \times 5)} = 400$$

In this case and in the tables quoted above, the sample size is independent of the total number of animals in the population. This is because prevalence is a proportion: the larger the study population the greater also the number of diseased animals.

Often the sample size is predetermined (for example, by the availability of animals), or the prevalence obtained from a survey is different from that which was expected. In all cases, the confidence limits (that is, the precision) of the prevalence value obtained from the survey will need to be calculated. This can be calculated by solving the above formula for L.

If, for example, after sampling 400 birds the prevalence was found to be 70%, the accuracy of the estimate would be:

$$L = \sqrt{\left(\frac{(4 \times 30 \times 70)}{400}\right)} = \sqrt{21} = 4.6$$

Thus, the estimated prevalence would be between 65.4% and 74.6%.

2. Sampling from a small (finite) population

The table and the calculation above are acceptable if the study population is large in relation to the sample. For statistical reasons, in small populations it is possible to select a smaller sample than from theoretically infinite populations and achieve the same degree of precision.

The required sample size, n*, is given by the following formula:

$$n^* = \frac{n}{\left(1 + \frac{n}{N}\right)}$$

where n is the sample size, based on an infinite population and obtained from the previous calculation; N is the size of the study population.

Thus, for the above examples, if the population size (number of chickens in a village) is 500, the sample size needed would be:

$$n^* = \frac{2500}{\left(1 + \frac{2500}{500}\right)} = \frac{2500}{6} = 417 \text{ for +/–2\% accuracy}$$

or

$$n^* = \frac{400}{\left(1 + \frac{400}{500}\right)} = \frac{400}{1.8} = 223 \text{ for +/–5\% accuracy}$$

A table of sample sizes required to estimate prevalence (based on an expected prevalence of 50%) can be constructed as follows:

Flock size	Accuracy of estimate (+/– %)			
	1	2	5	10
100	99	96	80	50
200	196	185	133	67
300	291	268	171	75
400	385	345	200	80
500	476	417	222	83
600	566	484	240	83
700	654	547	255	88
800	741	606	267	89
900	826	662	277	90
1000	909	714	286	91

Round up these values to give a bit more flexibility with the estimate.

4.9.2 Determining vaccine effectiveness

In order to measure whether vaccination has been effective, birds could be sampled at a rate sufficient to determine whether 80% of birds had a protective titre, with an accuracy of +/– 10%.

NOTE:

Epidemic theory suggests that if at least 70% of a population is immune, then disease outbreaks are unlikely to occur because there are not enough susceptibles to propagate an epidemic. Eighty per cent +/– 10% gives the ability to determine if more than 70% of the population have protective titres.

The following number of birds would then need to be sampled (sample sizes have been rounded up):

Flock size	Sample size
100	40
200	50
300	55
400	55
500	60
600	60
700	60
800	60
900	60
1000	60

If more than 80% of the sample had protective titres, it would be reasonably certain (95% probability) that the population from which the sample was drawn was adequately protected. If less than 80% of the sample did not have protective titres it could be surmised that the population was only partly protected.

If it is not possible to get a good random sample, collect samples at the above rate, but use a cut-off for vaccine effectiveness at 90% of the sample with protective titres.

4.9.3 Exercises

Exercise 1: How many birds do you need to bleed from a flock of 50 vaccinated birds to determine the level of flock immunity? From a flock of 100 birds, 200 birds?

Exercise 2: Field staff have contacted you saying that birds have been vaccinated using out-of-date vaccine by mistake and that no vials from that batch are available for testing. How would you deal with this situation?

Exercise 3: Staff from a provincial laboratory performed an ND seroprevalence survey and found that only 1% of birds were positive for antibodies to ND. Field veterinarians had indicated that ND was a major problem. How would you interpret the results?

5.0

Practical aspects of I-2 ND vaccine distribution

5.1 General recommendations for freeze-drying I-2 ND vaccine

Freeze-drying (lyophilisation) is a way of preserving a biological product that increases its storage life and portability by reducing its rate of degradation. Since water and oxygen play an important role in degradation, these are removed from the product during freeze-drying without disrupting its structure. When done correctly, freeze-drying should not significantly reduce the infectivity (potency) or stability of the virus or vaccine.

Inefficient freeze-drying will cause significant loss of titre or problems with the moisture content of the vaccine. High moisture content will result in poor stability of the vaccine, and excessive drying may result in inactivation of the virus.

Since the method of operation will vary from one type of freeze-drying unit to the next, only general aspects of freeze-drying will be considered here. For a description of the freeze-drying process, see Mariner (1997) and the instructions accompanying the freeze-drying unit.

In general, ensure that:

- there are enough trained and reliable staff to operate the freeze-drying unit
- staff are rotated during busy times
- there are funds to pay staff overtime (freeze-drying is a long process and will require out-of-hours work)
- the freeze-drying unit is calibrated and serviced regularly
- only one type of vaccine is ever freeze-dried at a time
- routine procedures such as cleaning and vacuum checks are carried out **before** a freeze-drying run.

5.2 Inspection of vaccine after freeze-drying

After freeze-drying, vials of vaccine should be inspected for:

- the integrity of vacuum — the rubber stoppers should be held firmly into the vial, and the top of the stopper should show a depression if it has been sealed under vacuum
- the colour of freeze-dried pellet — the vaccine pellet should be the same colour throughout
- the consistency of freeze-dried pellet — the vaccine pellet should be compact (like a tablet) and homogeneous after dilution
- the presence of foreign bodies.

If the pellet is uneven in colour or shows signs of boiling, then the freeze-drying has been done incorrectly and vaccine stability and infectivity will be affected. Vaccine should be discarded.

The residual moisture content of freeze-dried vaccine should be measured (VICH 2002). Excess residual moisture in the vaccine will influence the stability of the vaccine and the length of time it can be stored. The integrity of the vacuum seal should also be confirmed (Allan, Lancaster and Toth 1978).

After inspection, vials are sealed using aluminium closures. Where the laboratory produces other vaccines in addition to I-2, the use of coloured caps or closures should be considered.

5.3 Stability testing of vaccine

Stability tests provide evidence of how the quality of a vaccine varies with time under different environmental conditions. Data generated from such tests allows storage conditions and shelf life of the vaccine to be established. Tests should cover those features that may change during storage and are likely to influence the quality, safety and/or efficacy of the product. The length of the studies and the type of conditions should be sufficient to cover storage, transport and subsequent use of the vaccine.

Many factors influence the stability of the vaccine. For instance, the stability of freeze-dried I-2 ND vaccine depends on:

- the nature of the stabiliser added to the vaccine
- the efficiency of the freeze-drying process
- the air and moisture tightness of the rubber stopper
- the quality of the freeze-dried pellet of vaccine
- the temperature of storage and transport of the vaccine.

A change in any one of these factors may influence the quality of the vaccine. Therefore, although test data on I-2 ND vaccine produced at another laboratory may serve as a guide, each manufacturer must perform stability tests on the vaccine produced in their own laboratory.

The one factor that most affects vaccine stability in many developing countries is temperature. Where the cold chain is inadequate or non-existent, vaccine will rapidly lose its potency. Under such conditions, vaccination may fail to protect chickens and farmers will lose confidence in the vaccine. To prevent this happening, the stability of I-2 ND vaccine must be tested at various temperatures.

Stability tests are simple to perform. Vaccine is held at:

- the normal storage temperature (ideally 2–8°C)
- room temperature (both summer and winter if there are significant seasonal variations)
- 37°C

and the potency of the vaccine is measured by titration (see Section 4.4) at specified intervals. For freeze-dried vaccine, testing should be performed once each month (vaccine stored at 2–8°C), once in every one to two weeks (room temperature), and twice each week (37°C). 'Wet' vaccine kept at room temperature and 37°C may need to be tested every day.

Knowledge of the stability of the vaccine at 2–8°C will allow manufacturers to determine the shelf life of the vaccine and set a realistic expiry date. Expiry dates should always be set to allow for a margin of misuse. For example, if testing shows that the vaccine retains its infectivity titre for 15 months if held at 2–8°C, then the expiry date should specify 12 months.

National registration authorities will probably require stability data for three batches of vaccine. For the purposes of shelf life determination, they may allow accelerated stability testing. These are studies designed to increase the rate of degradation of the vaccine by using exaggerated storage conditions — for example, vaccine held at 37°C.

5.4 Labelling vaccine

Label the vaccine as soon after production as possible. This will avoid mix-up with other batches of I-2 ND vaccine or other types of vaccines in storage.
The following information should be printed on the label (Figure 16):

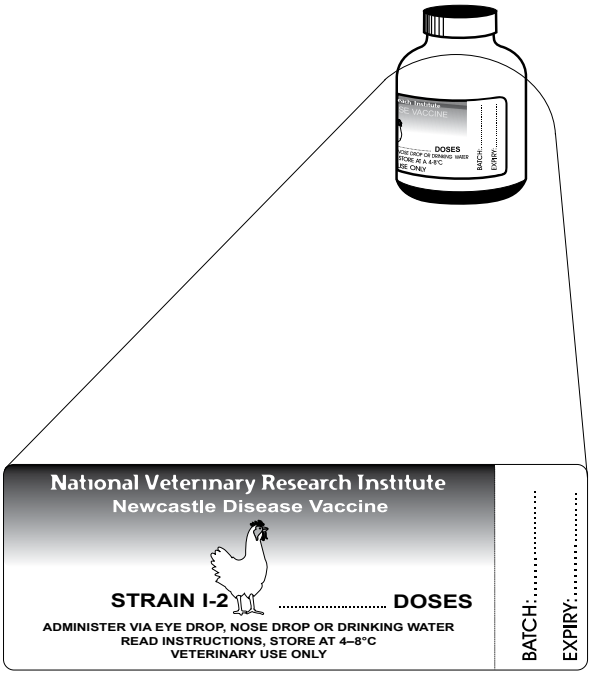


Figure 16: *The label on the vaccine vial contains very important information.*

- the name of the vaccine, including the strain of virus used
- the disease the vaccine will prevent
- the route of administration
- the storage conditions
- the batch number
- the expiry date (both month and year, calculated from vaccine stability trials)
- the number of doses in the container
- the name (and address) of the manufacturer
- the registration or licence number
- 'For animal use only'
- a picture of a chicken.

Additional information could include:

- the composition of the vaccine
- the name of any bacteriostatic agents added.

For freeze-dried vaccine, the expiry date is set from the date of manufacture, taken as the date of freeze-drying. It is important that both the month and year of manufacture be included. Always allow a reasonable margin of error! For example, if stability testing shows that the vaccine retains its titre for 15 months if held at 2–8°C, then the expiry date should specify 12 months.

To ensure that the vaccine is used properly, an instruction leaflet describing the handling, reconstitution and use of vaccine should be provided with the vaccine. Where possible, the instructions should be written in the language most commonly spoken by the people who will administer the vaccine. The following information should be given on the leaflet:

- the name of the vaccine
- the registration number
- the composition of the vaccine (including the strain of virus used, 'prepared in eggs', minimum titre per dose)
- the indications for the use of the vaccine ('To prevent ND in village chickens', 'Safe for chickens of all ages', 'Vaccinate before an outbreak is expected')
- the route of administration
- the precautions (such as 'Do not vaccinate sick animals'; 'For animal use only')
- the storage conditions
- presentation (number of doses per vial, freeze-dried or 'wet' vaccine)
- the withholding period
- the name and address of the manufacturer
- contacts for further information and instructions.

Always check with the national registration authority before printing labels and leaflets in large numbers. They should provide details of the information to be included on the vaccine label and on any leaflet that accompanies it.

Vaccine manufacturers may also wish to prepare a summary of this leaflet for use by field staff, listing the basic instructions for the administration of I-2 ND vaccine. Examples are shown in Appendix 9.

5.5 Storing vaccine

I-2 ND vaccine must be stored away from excess heat, cold or light. Fluctuating temperatures should also be avoided. Both freeze-dried and 'wet' vaccines are best stored in a cold room or refrigerator at 2–8°C. A number of containers or vials from each batch of vaccine released for public use should be retained and stored by the manufacturer until after the expiry period has ended. These are called retention samples and will be useful if complaints are received about the quality of the vaccine.

Storage areas should be clean and dry. They should be regularly checked and the temperature monitored. A maximum–minimum thermometer should be placed in each cold room and the temperatures written into a record book on a daily basis.

The 'critical' temperature, that is the temperature above which vaccine quality may be affected (normally 10°C), and the person responsible for taking action should this temperature be reached must be nominated. Monitoring should be assigned to a responsible staff member.

Vaccine that has been exposed to high or fluctuating temperatures will lose its potency and should not be used. Potency will not be restored by returning vaccine to the correct storage temperature.

5.6 Maintaining the cold chain

To ensure optimal potency, vaccine must be stored and handled correctly at all times. The process of production, distribution and use of vaccine is composed of a number of steps, each of which is a link in what is known as 'the cold chain'. The cold chain is an essential component of any vaccination program since if any one of these links is broken, the vaccine may lose potency and will fail to provide protection. There are three major elements in the cold chain:

- equipment – for storage and transport of vaccine
- personnel – to use, monitor and maintain the equipment and carry out the vaccination
- procedures – to ensure the correct conditions of storage, transportation and use of the vaccine.

Figure 17 shows the links in a typical I-2 ND vaccine cold chain in Mozambique and what may happen if links in the chain are broken.

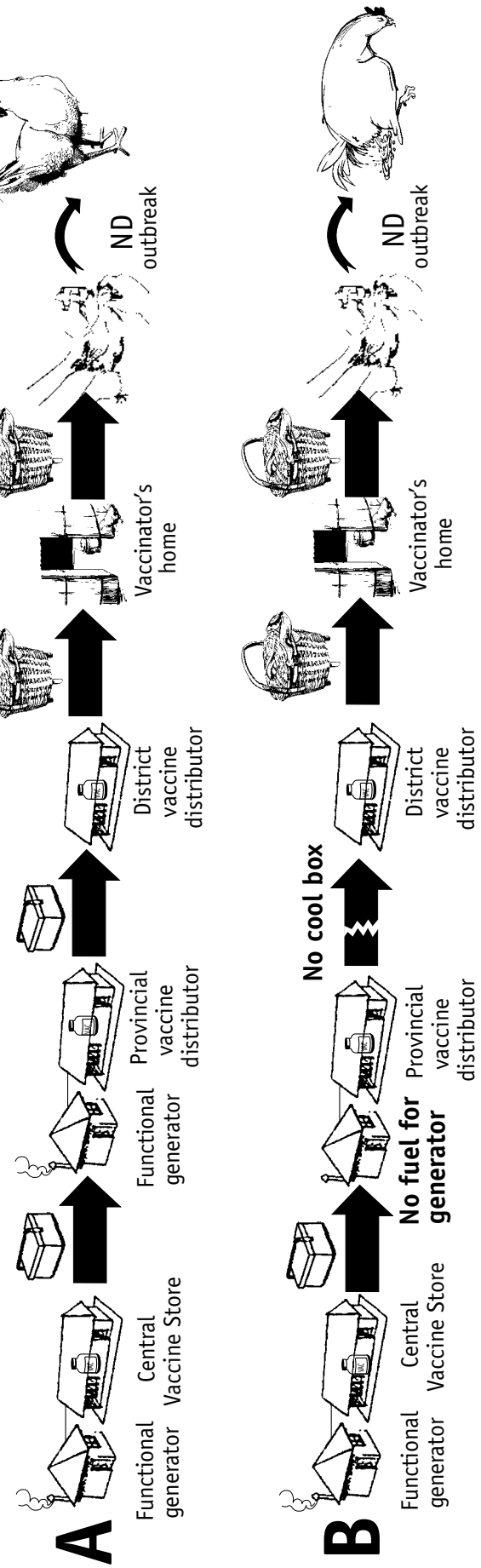


Figure 17: *A typical cold chain encountered in Mozambique.*

1. **Care during vaccine production.** Ensure correct storage of master seed and working seed, of allantoic fluid before freeze-drying and correct operation of freeze-dryer.
2. **Storage of vaccine at the Central Vaccine Store.** Ensure that vaccine is kept at a suitable temperature, that the temperature is monitored daily, that the person responsible for monitoring the temperature knows what to do if the temperature exceeds the set limits and that alternative arrangements are in place in case of equipment breakdown.
3. **Dispatch and transport of vaccine.** Ensure that the vaccine is packed in an insulated container with ice or freezer bricks and that the handling instructions are on the outside of the box. Make sure that instructions on how to store, dilute and use the vaccine are included in the container. During transport, ensure that the vaccine package is kept in the cool and is not in direct sunlight. If it is sent by commercial transport, ensure that instructions on how to take care of the vaccine are on the outside of the box, and that a contact name and number are given to the transporters. Vaccine should reach its destination in as short a time as possible.
4. **Storage at the provincial and district vaccine distribution points.** Ensure that vaccine is kept at a suitable temperature, that the temperature is monitored daily, that the person responsible for monitoring the temperature knows what to do if the temperature exceeds the set limits and that alternative arrangements are in place in case of equipment breakdown.
5. **Dispatch of vaccine from the provincial and district vaccine distribution points.** If an insulated box and ice or freezer bricks are not available to take vaccine to the field, evaporative cooling will keep it cool for a short time. Wrap the vaccine in a damp cloth and place it in an open-weave basket. The movement of air through the basket and over the cloth will cool the vaccine.
6. **Storage and handling in the field.** Store the vaccine in a cool place. Until the vaccine is ready for use, it could be stored at home beside a clay water pot in the shade. When transporting the vaccine in the field, use an open-weave basket as described above. Use reconstituted freeze-dried vaccine for no more than two days after reconstitution.

Make sure that every person in the cold chain — vaccine producers and distributors, and community vaccinators — understands the need for maintaining the cold chain and their role in maintaining it.

NOTE:

- Vaccine should always be transported in accordance with national and international regulations. Check with the postal or transport authorities for the correct procedures.
- When planning vaccination campaigns, good coordination is needed between laboratory staff, distributors of the vaccine (for instance, veterinary pharmacies, NGOs or provincial laboratory staff) and community vaccinators. This will ensure that good quality vaccine reaches the vaccinators when it is needed.
- Rapid, reliable means of communication and transport are needed to ensure that vaccine quality is maintained during its journey from producer to vaccinator.
- Always record the date on which the vaccine leaves the cold chain, preferably on the label of the vaccine vial. It is useful to 'recall' vaccine from the field from time to time, or to request the return of unused vials kept under dubious conditions. The potency of the vaccine can then be tested by titration and the effectiveness of the cold chain checked.

5.7 Packaging vaccine

For distribution, I-2 vaccine must be dispensed into suitable containers. Containers must be:

- 'virus friendly'
- chemically and physically inert
- low cost
- of appropriate size (number of doses).

Cost is a major consideration, especially when freeze-dried vaccine is being produced. The major component of the cost of freeze-dried I-2 vaccine is the cost of the container — glass vial, rubber stopper, label and aluminium seal. The cost of the container remains the same whether it holds 20, 50 or 100 doses of vaccine.

5.7.1 Freeze-dried vaccine

Good quality containers are required for freeze-dried vaccine to ensure uniform drying, a good seal and maintenance of the vacuum.

Glass vials should be of good quality glass with thin walls of uniform thickness. Vials should not be recycled since complete cleaning is virtually impossible and contamination of the neck may result in the development of micro-leaks during storage. This will cause problems with the stability of the vaccine. Before use, vials must be stored in a dust-free environment, rinsed with distilled water and sterilised in a hot air oven.

The closures (bungs or stoppers) used for freeze-drying are generally made from natural rubber or a synthetic compound such as butyl or neoprene. They must be chemically inert and have appropriate slotting to allow removal of moisture during freeze-drying. Before use, they should be sterilised in an autoclave at 121°C. It is necessary to dry stoppers at 135–142°C for four hours after autoclaving since they may absorb moisture during autoclaving. If the stoppers are not dried, correctly-dried vaccine may absorb moisture from the stopper during storage, leading to a loss of stability.

Aluminium seals are used to hold the rubber stopper in place and to give the user of the vaccine confidence that the vaccine has not been tampered with. If a variety of vaccines is manufactured, different coloured seals may be used to differentiate the various vaccines.

Further information may be found in Srinivasan, Kariath and Bangar Raju (1997).

5.7.2 'Wet' vaccine

A wide range of containers is available for wet I-2 ND vaccine. Plastic containers are the most frequently used. These are lighter, less fragile and potentially cheaper than glass containers. Vaccine may also be transported in ready-to-use plastic eye-droppers or disposable transfer pipettes, containing just enough doses for a farmer's flock. Plastic eye-droppers with tamper-proof caps are ideal.

5.7.3 'Virus-friendly' vaccine containers and eye-droppers

Plastic containers used to store or administer I-2 ND vaccine must be tested before they are used to show that they are 'virus-friendly'. The containers could be contaminated with chemical residues that will harm the virus, or be made of a type of plastic that will inactivate the virus, and therefore the vaccine. Such containers or droppers must not be used. Before ordering a new batch of containers or eye-droppers, ask the supplier for a few for testing.

Equipment and materials

Vaccine container or eye-dropper bottle and tip
I-2 vaccine
Sterile PBS with antibiotics
10 mL sterile pipette or syringe

Procedure

1. If using freeze-dried vaccine, select the batch of vaccine to be tested, take a sample vial and allow it to reach room temperature.
2. Dilute the vaccine with a suitable volume of PBS. This need not be the recommended volume for dilution.
3. Transfer half of the diluted freeze-dried or 'wet' vaccine into the container or eye-dropper, and seal.
4. Seal the vaccine vial containing the remainder of the vaccine.
5. Place the vial and container or eye-dropper in a dark, cool place[1] for 24 hours.
6. After 24 hours measure the infectivity titre of both samples of vaccine by egg inoculation (see Section 4.4 for the procedure) and compare.

The containers or eye-droppers are suitable for use if the infectivity titre of the vaccine stored in the container or eye-dropper is equal to the titre of the vaccine stored in the vaccine vial.

5.7.4 Drop size

The size of the drop delivered by the eye-dropper should also be checked. Eye-droppers that are made from low density polyethylene (LDPE) and deliver 35–40 drops per mL are recommended. Methods of calibrating eye-droppers are described in Appendix 3 of *Controlling Newcastle disease in village chickens: a field manual* (Alders and Spradbrow 2001).

Equipment and materials

Eye-dropper bottle and tip
Water
1 mL pipette or syringe

Procedure

1. Remove the tip from the eye-dropper.
2. Place 1 mL of water into the eye-dropper.
3. Invert the eye-dropper, squeeze gently and count the number of drops.
4. Repeat at least two more times.

The eye-droppers are ideal for use if the number of drops delivered per mL is 35 to 40.

[1] This procedure tests the infectivity of vaccine kept at room temperature, the temperature at which vaccine is likely to be kept under field conditions. A more correct measure of the capacity of a container to inactivate virus would be to place both the glass vial and the container at 4°C for 24 hours.

5.8 Vaccine transport

All transport containers should be tested for their effective 'cold life' — that is, the number of hours that the vaccine will be kept at a safe temperature (below 10°C) after removal from the refrigerator or cold room, when packed with the recommended number of frozen ice or gel packs. The cold life depends on:

- the type of cool box or container — the insulation material, its thickness and the method of construction of the container
- the number, temperature of freezing and position of ice or gel packs placed in the cool box
- the surrounding temperature
- how many times the cool box is opened, and for how long.

Data loggers

A data logger is a piece of electronic equipment that is used to record data such as temperature or humidity. It is like a cassette recorder, but instead of recording sound, it records temperature at intervals you have set using a computer and software. The data logger will continue to record and store information until you stop it. The information can then be downloaded, displayed and analysed. Before computers, chart recorders were used to record this information and before chart recorders, a thermometer, pencil, paper and a dedicated technician!

Dataloggers are useful in the laboratory for monitoring the temperature of equipment such as egg incubators, cold rooms and refrigerators and for carrying out simple experiments on the cold life of vaccine containers. A datalogger is not expensive and may save many times its purchase price in ensuring that correct transport conditions for vaccine are determined.

Commercially available freezer bricks or gel packs are ideal for keeping vaccine cool. However, these are expensive and generally will not be returned to the vaccine producer or distributor after the vaccine is used. Therefore, the cost should be added to the cost of the vaccine. Frozen disposable plastic mineral water bottles, three-quarters filled with water or saturated salt solution, are a low cost, readily available alternative. However, these may not maintain low temperatures as long as the commercially available packs.

5.9 Reconstituting and administering I-2 vaccine by eye-drop

Vaccine should be reconstituted and administered in a shady area, out of direct sunlight. This will ensure the vaccine maintains its quality as long as possible.

5.9.1 Reconstituting and administering freeze-dried I-2 vaccine

Equipment and materials

10 mL syringe
Vial of I-2 ND vaccine
Eye-dropper bottle and tip
Diluent provided by vaccine manufacturer or boiled potable water (see Appendix 9)

Procedure

1. Remove the aluminium seal and rubber stopper from the vaccine vial.
2. Measure the recommended volume of diluent or potable water into a syringe and add to the vaccine vial.
3. Replace the rubber stopper and mix the vaccine by gently shaking the vial.
4. Pour the contents of the vaccine vial into the clean eye-dropper.
5. Put the tip of the eye-dropper back onto the bottle and swirl to mix the vaccine.
6. Ask your assistant or the owner of the bird to hold the chicken to be vaccinated horizontally, with one eye facing you.
7. Hold the head of the bird and open the eye with the thumb and forefinger of one hand.
8. Hold the eye-dropper vertically and squeeze it gently to allow one drop of vaccine to fall into the chicken's eye. Make sure that the drop of vaccine spreads over the surface of the eye before releasing the bird.

5.9.2 Administering wet I-2 vaccine using an eye-dropper

1. Ask your assistant or the owner of the bird to hold the chicken to be vaccinated horizontally, with one eye facing you.
2. Hold the head of the bird and open the eye with the thumb and forefinger of one hand.
3. Hold the eye-dropper vertically and squeeze it gently to allow one drop of vaccine to fall into the chicken's eye. Make sure that the drop of vaccine spreads over the surface of the eye before releasing the bird.

NOTE:

- Where freeze-dried vaccine is reconstituted with local potable water, use the vaccine for two days only. Any unused vaccine must be discarded on the third day because the potency of the vaccine may have been affected by elements in the water, or the vaccine may have been exposed to field contaminants on opening.
- Chlorinated tap water is unsuitable for reconstitution or dilution of I-2 ND vaccine. If it is the only water available, allow it to stand overnight (Alders and Spradbrow 2001) before use.

5.10 Troubleshooting

Use problems that arise as opportunities to learn and to improve techniques. If a problem occurs, the laboratory supervisor and staff should troubleshoot the problem — that is, discuss it and work through the following steps to find a solution:

- Identify the problem.
- Review the procedure step by step, including the records and calculations.
- Identify where the problem may have occurred.
- Work out what could have caused the problem.
- Think of what can be done to confirm the cause of the problem.
- Identify steps that can be taken to reduce the chances of the problem occurring again.
- If necessary, review and revise the standard operating procedures.

Remember

- Always read the instructions and protocol first. This way you will be sure to use the correct procedure.
- Always record all details of your work. When problems occur, you will be able to check data such as weights, dilutions and calculations.
- Good records save money! Problems can be more readily identified if all aspects of the work are recorded. Remember that the sale price of your vaccine must cover not only the cost of production of the batches of vaccine that are sold, but also the cost of the batches that are rejected.

Some of the problems that may be encountered during vaccine production are shown in Figure 18 and discussed in the examples.

Example 1

Fertile eggs are procured from the usual source and held at 16°C for 48 hours before being placed in the incubator. They are incubated for nine days then taken from the incubator and candled in preparation for inoculation of working seed virus. However, approximately 35% of the eggs must be discarded since they contain dead embryos.

❑ Problems with embryo viability before inoculation could indicate poor quality embryos (problems with health of parent flock), incorrect holding temperature or humidity before incubation, or incorrect temperature and humidity during incubation. Interruptions to the power supply to the incubator could also cause poor embryonic viability.

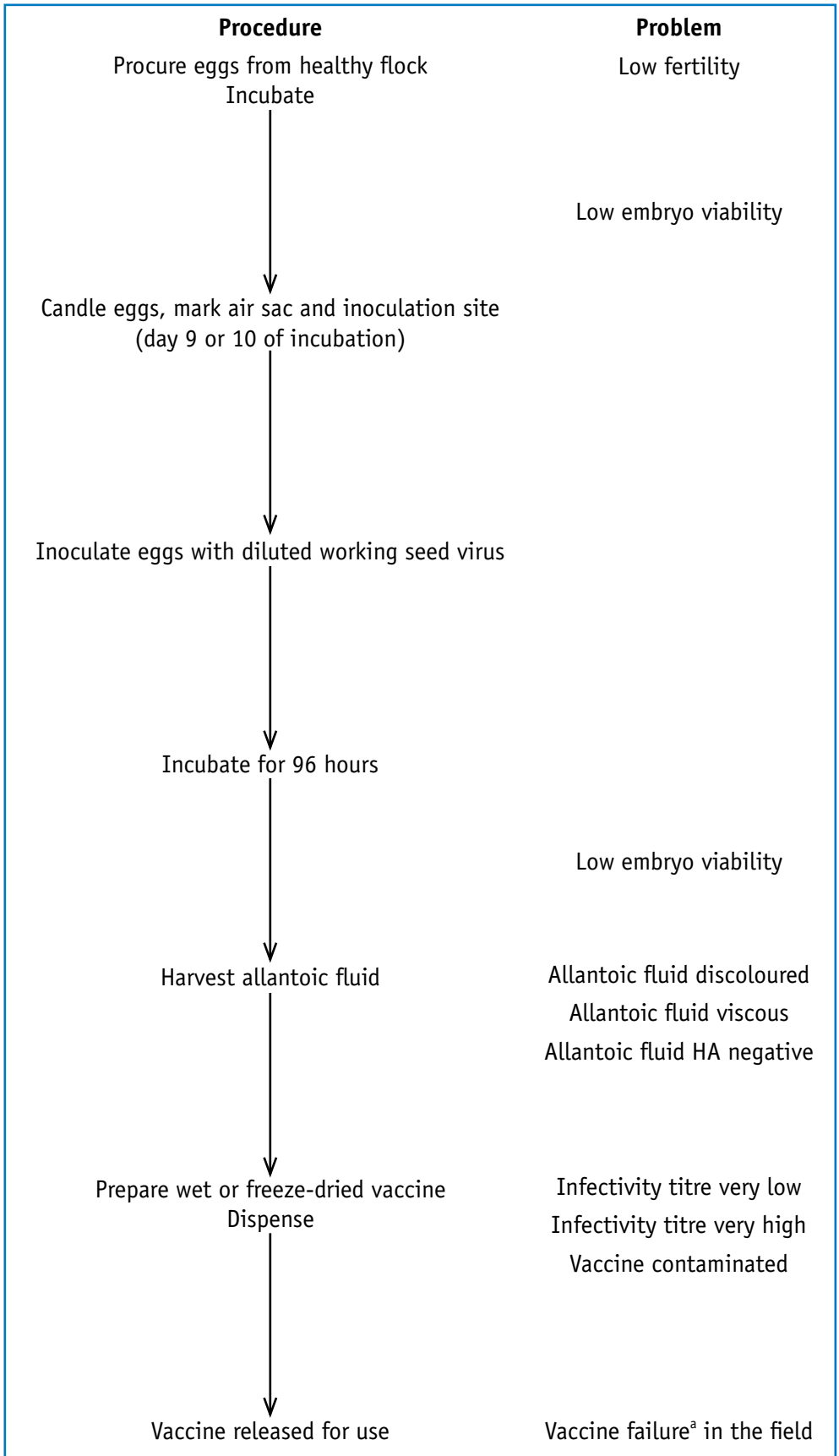
➢ Review previous records of embryonic viability during incubation and if necessary contact the egg supplier to ask if any health problems or changes in management have occurred in the parent flock. Use a thermometer or data logger to monitor the temperature of the incubators and ensure that the water trays inside the incubators have been filled regularly. If possible, find out if the power supply was interrupted and for how long. If the area experiences frequent and long-lasting interruptions to the electricity supply, it may be necessary to provide a back-up power supply.

Example 2

100 ten-day-old embryonating eggs are candled and inoculated with I-2 ND working seed virus. After 24 hours of incubation, the eggs are candled again and 15 show evidence of embryonic death.

❑ Embryonic death in more than 2% of inoculated eggs within 24 hours of inoculation usually indicates faulty technique — for example, the inoculation site was incorrectly marked or the needle used for inoculation was too large or too long, so that the needle penetrated the yolk sac or large blood vessels (if deaths occurred after 24 hours incubation, then the problem is generally due to bacterial contamination of the inoculum).

➢ Review the candling and marking of eggs for inoculation. Check the gauge and length of the needles used for inoculation. Check the inoculation technique by mixing a little blue food dye with PBS and inoculating a few eggs using the usual route and technique. Open the eggs carefully after cooling in the refrigerator and look at the distribution of the blue dye. I-2 ND virus will kill embryos inoculated via the yolk sac.



[a] Some of the reasons for vaccine failure are discussed in Appendix 8 of *Controlling Newcastle disease in village chickens: a field manual* (Alders and Spradbrow 2001).

Figure 18: *Flow chart of I-2 vaccine production showing some of the problems that may be encountered during I-2 ND vaccine production and field use.*

Example 3

Tenfold serial dilutions of freshly prepared wet I-2 ND vaccine are prepared and inoculated into embryonating eggs. The results of the infectivity titration are shown below.

Dilution of Vaccine	Result
10^{-6}	+ + + + +
10^{-7}	+ + + + +
10^{-8}	+ + + + +
10^{-9}	+ + + + +
10^{-10}	+ + + + +
10^{-11}	+ + + + +

- ❑ These results suggest that the vaccine has an infectivity titre greater than that usually achieved with freshly prepared I-2 ND vaccine. This is an error due to faulty technique and is generally due to failure to change pipette tips or pipettes when preparing the dilutions of vaccine for inoculation. However, it may also be due to contamination of PBS (used as diluent) with I-2 ND virus or another organism that causes haemagglutination.
- ➢ Repeat the titration and ensure that a new pipette or pipette tip is used at each dilution (see Section 4.4). If the result is the same, then it is likely that the PBS is contaminated. To test for contamination of the PBS, inoculate several eggs with PBS, incubate for 96 hours and test the allantoic fluids for the presence of virus using the HA test (see Section 4.3.1).

Example 4

Two vials of I-2 vaccine (250 doses per vial) have been returned from the field as field staff were concerned that the vaccine was provoking high mortality among vaccinated birds.

- ❑ I-2 ND vaccine is safe and will not harm birds even if 100 times the recommended dose is given. Problems may occur where counterfeit or mislabelled vaccine is used, or where birds are vaccinated in the face of an outbreak. Appendix 8 of *Controlling Newcastle disease in village chickens: a field manual* (Alders and Spradbrow 2001) discusses some of the reasons for vaccine failure.
- ➢ Check the label of the vaccine vial. Titrate the vaccine. (When the vaccine was titrated the titre was above the recommended minimum and no embryonic death was recorded during the infectivity titration).
- ➢ Ask the field staff about the situation in the field. Were any of the birds sick at the time of vaccination? Were any problems experienced during vaccination? How was the vaccine prepared? Were other birds in the village healthy? (These investigations revealed that the vaccine had been administered to birds in a flock two days after the neighbour's flock had died of ND.)
- ➢ Remind the field staff that it takes 7–14 days for birds to develop a protective antibody titre after vaccination. Sick birds or those in contact with birds that have died from ND should not be vaccinated as farmers will associate mortalities with the vaccine rather than the disease.

Example 5

Two plastic droppers containing wet I-2 ND vaccine from a box of 300 sent to the field have been returned to the laboratory because the box had been left outside the cool room for one week by mistake. The field staff would like to know if they can still use the remaining vaccine. Each dropper contains 30 doses and produces a drop size of 40 µL.

➢ Titrate the vaccine. Based on the results, decide if the field staff should use the vaccine.

The vaccine was titrated and yielded the following results. Is the vaccine acceptable for use? (See Appendix 11 for the answer.)

Dilution	HA results	No. of infected eggs	No. of uninfected eggs	Accumulated total infected eggs	Accumulated total uninfected eggs	Ratio and % accumulated infected eggs
10^{-1}	+ + + + +					
10^{-2}	+ + + + +					
10^{-3}	+ + + + +					
10^{-4}	+ + + + +					
10^{-5}	+ + + + +					
10^{-6}	+ + + + +					
10^{-7}	+ + + + +					
10^{-8}	+ + + + –					
10^{-9}	+ + + – –					
10^{-10}	– – – – –					

➢ Review the cold chain. Remind the field staff of the conditions under which vaccine should be stored to best conserve vaccine infectivity.

6.0

Newcastle disease diagnosis

A provisional diagnosis of ND in the field is based on the history of the disease outbreak, the clinical signs and the lesions observed at necropsy. The clinical signs of ND vary widely and are dependent on the strain of the virus, the age of chicken, the presence of concurrent infections, the immune status of the host and environmental stress, among others. Clinical signs associated with ND include respiratory distress, diarrhoea, depression, oedema of the head, face and wattles, nervous signs and death. In laying birds, egg production may also be reduced and misshapen eggs may be produced (Alders and Spradbrow 2001). Lesions observed at necropsy of birds that have died due to ND are not pathognomonic.

In order to confirm the diagnosis, ND virus must be isolated, identified and its pathogenicity determined in the laboratory. In most laboratories, the assessment of virulence will be based on in vivo testing of the isolate. However, the current OIE definition of what constitutes ND for the purposes of trade, control measures and policies allows molecular assessment of virulence (OIE 2011b).

OIE definition for reporting an outbreak of ND

Newcastle disease is defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:

a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (*Gallus gallus*) of 0.7 or greater.

or

b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 and 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterisation of the isolated virus by an ICPI test.

In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene, 113-116 corresponds to residues –4 to –1 from the cleavage site.

(OIE 2011b)

ND is confirmed by isolation of the virus in embryonating chicken eggs and identification and characterisation of virulent ND virus. Such isolates will provide virus for use in trials testing the efficacy of I-2 ND vaccine if a suitable challenge strain is not available. Collection and storage of field strains of ND virus is important for epidemiological studies.

6.1 Virus isolation and characterisation

Collect samples of lung, spleen, liver, heart, brain and marrow from a long bone (for example, femur or humerus), wrap in plastic and place in a cool box with ice or freezer bricks. Other samples collected should reflect clinical signs.

For example, the brain (or whole head) should be collected if nervous signs were observed, as well as any organs or tissues showing lesions. If it is possible, collect tracheal swabs, cloacal swabs and/or faeces since these samples are most consistently associated with successful virus isolation (Alexander 1998). Swabs should be fully immersed in antibiotic medium. Collect faeces into antibiotic medium.

When it is not possible to keep the samples cold or when it is uncertain that the samples will arrive at the laboratory within 24 hours, spleen, lung, the entire head (brain still within the head) and long bones should be collected into 50% glycerol (glycerin) in saline and kept as cold as possible (Alders and Spradbrow 2001).

The type and concentration of antibiotics used in transport media may vary according to the sample to be transported and the local availability and cost of antibiotics. High concentrations of antibiotics are needed in transport medium for faecal samples. Suggested levels in PBS are 10 000 IU/mL penicillin, 10 mg/mL streptomycin, 0.25 mg/mL gentamycin and 5000 IU/mL nystatin (Alexander 1998). For transport of swabs, the concentration of antibiotics may be reduced: PBS with 2000 IU/mL penicillin, 2 mg/mL streptomycin, 0.05 mg/mL gentamycin, and 1000 IU/mL nystatin at pH 7.0–7.4 is recommended.

Try to keep samples cool during transport to the laboratory. High temperatures and decomposition of tissues destroy virus infectivity.

For isolation of virus, it is ideal if samples are kept separate. However, in the laboratory it is often necessary to pool organ and tissue samples.

Swabs in antibiotic medium

1. Vortex the sample to free as much sample material as possible from swab fibres.
2. Remove the swab and transfer the sample to a centrifuge tube. Centrifuge at 1000–1500 × *g* for 10 minutes in a refrigerated centrifuge at 4–10°C. This will sediment the tissue debris and most bacteria.
3. Remove the supernatant using aseptic technique and inoculate into embryonating eggs (see below Faeces: step 4).

Tissues and organs

1. Grind or mince the sample finely using a homogeniser or a mortar and pestle, or load the sample into a syringe and squirt it through a narrow gauge needle. The sample could also be ground using sterile glass beads or sand.
2. Make a 20% w/v suspension in antibiotic medium (PBS with 2000 IU/mL penicillin, 2 mg/mL streptomycin, 0.05 mg/mL gentamycin and 1000 IU/mL nystatin, pH 7.0–7.4).
3. Centrifuge at 1000–1500 × *g* for 10 minutes in a refrigerated centrifuge at 4–10°C to sediment tissue debris and most bacteria. Remove the supernatant aseptically and inoculate into embryonating eggs (see below Faeces: step 4).

Faeces

1. Faeces should be collected as a 20% w/v suspension in antibiotic medium (PBS with antibiotics at pH 7.0–7.4: 10 000 IU/mL penicillin, 10 mg/mL streptomycin, 0.25 mg/mL gentamycin and 5000 IU/mL nystatin).
2. Centrifuge at 1000–1500 × *g* for 10 minutes in a refrigerated centrifuge at 4–10°C to sediment tissue debris and most bacteria.
3. Remove the supernatant aseptically.
4. Inoculate 0.1 mL of the supernatant into the allantoic cavity of 9- to 10-day-old embryonating chicken eggs.

5. Incubate the eggs at 37°C and candle each day. Place the eggs containing dead embryos in a refrigerator or cold room at 4°C.
6. Test the allantoic fluid from all eggs containing dead or dying embryos, and all eggs 5–7 days after inoculation for haemagglutinin.

Samples that are HA positive must undergo further tests, including titration of HA activity to confirm and quantify HA activity and identification of the HA agent by means of the HI test (Terregino and Capua 2009).

NOTE:

It is best to use eggs from an SPF flock or a flock free of NDV antibodies, but if these are not readily available good quality eggs from a healthy minimum disease flock may be used.

6.2 Pathogenicity tests

It is necessary to confirm the virulence of ND virus isolates using tests for pathogenicity. This will differentiate ND virus isolates of high and low virulence. Several tests for pathogenicity have been developed and these are described in detail in Alexander (1998). The three most commonly used are:

- mean death time (MDT) in eggs
- intracerebral pathogenicity index (ICPI)
- intravenous pathogenicity index (IVPI).

In addition, molecular studies have recently been used to assess pathogenicity. These rely on deducing the sequence of amino acids around a particular site by reverse-transcriptase-polymerase chain reaction (RT-PCR) and sequencing of the PCR product.

Only MDT will be described here since this is the easiest and cheapest way of estimating the virulence of an ND virus isolate and can be performed using the simple equipment found in most laboratories.

Mean death time (MDT) in eggs

1. Make a series of tenfold dilutions of the isolate in sterile isotonic saline.
2. Inoculate 0.1 mL of each dilution into the allantoic cavity of each of at least five 9- to 10-day-old embryonating chicken eggs.
3. Keep the remainder of the diluted isolate at 4°C.
4. Around 8 hours later, inoculate another 5 eggs at each dilution.
5. Incubate the eggs at 37°C and candle twice daily for 7 days.
6. Record the time at which each embryo is first observed as dead.
7. Calculate the minimum lethal dose. This is the highest dilution at which all eggs die.
8. Calculate the MDT. This is the mean time (in hours) for the minimum lethal dose to kill embryos.

Three groups are differentiated on the basis of MDT in SPF eggs (Hanson and Brandly 1955):

— velogenic <60 hours
— mesogenic 60–90 hours
— lentogenic >90 hours

If SPF eggs are not available, commercial eggs can be used to give an estimate of the MDT. These eggs will probably contain antibodies to ND, but the antibodies will be confined to the yolk sac until around day 15 of incubation (Senne 1998).

Initially, the virus will replicate in the cells lining the antibody-free allantoic sac. Virulent virus will then spread to the amniotic cavity and the embryo itself, killing the embryo. In contrast, avirulent ND virus will be confined to the allantoic cavity since it lacks the enzymes necessary to invade further.

MDT correlates well with the clinical disease that results from infection of susceptible chickens. Any isolate of ND virus that kills embryos in commercial eggs in less than 60 hours is definitely velogenic and suitable for use as a challenge strain.

7.0

Bibliography

Albiston, H.E. and Gorrie, C.J. 1942. Newcastle disease in Victoria. Australian Veterinary Journal, 18:75–79.

Alders, R.G., Fringe, R. and Mata, B.V. 2001. Characteristics of the I-2 live thermostable Newcastle disease produced at INIVE. In: Alders, R.G. and Spradbrow, P.B., ed. SADC Planning Workshop on Newcastle Disease Control in Village Chickens. Proceedings of an International Workshop, Maputo, Mozambique, 6–9 March 2000. ACIAR Proceedings No. 103:97–100.

Alders, R. and Spradbrow, P.B. 2001. Controlling Newcastle disease in village chickens: a field manual. ACIAR Monograph 82. 112pp.

Alders, R., dos Anjos, F., Bagnol, B., Fumo, A., Mata, B. and Young, M. 2002. Controlling Newcastle disease in village chickens: a training manual. ACIAR Monograph No. 86. 128pp.

Aldous, E.W. and Alexander, D.J. 2001. Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). Avian Pathology, 30:117–128.

Alexander, D.J. 1991. Newcastle disease. In: Rweyemamu, M.M., Palya, V., Win, T. and Sylla, D., ed. Newcastle Disease Vaccines for Rural Africa. Debre Zeit, Ethiopia, Pan African Veterinary Vaccine Centre. pp. 7–45.

Alexander, D.J. 1998. Newcastle disease virus and other avian paramyxoviruses. In: Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E. and Reed, W.M., ed. A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th edn, American Association of Avian Pathologists, Pennsylvania. pp. 156–163.

Alexander, D.J. 2000. Newcastle disease and other avian paramyxoviruses. *Office International des Epizooties*, Scientific and Technical Review, 19(2):443–462.

Alexander, D.J., Campbell, G., Manvell, R.J., Collins, M.S., Parsons, G. and McNulty, M.S. 1992. Characterisation of an antigenically unusual virus responsible for two outbreaks of Newcastle disease in the Republic of Ireland in 1990. The Veterinary Record, 130:65–68.

Allan, W.H. and Gough, R.E. 1974. A standard haemagglutination inhibition test for Newcastle disease (1) A comparison of macro and micro methods. The Veterinary Record, 95:120–123.

Allan, W.H., Lancaster, J.E. and Toth, B. 1978. Newcastle disease vaccines. FAO, Rome, Italy.

Amakye-Anim, J., Awuni, J.A., Coleman, T. and Seddor, V. 2000. Ghanaian trials with a locally produced thermostable Newcastle disease vaccine (strain I-2) in chickens. Paper presented at the Ghana Animal Science Association (GASA) 25th Animal Science Symposium on Sustaining Ghana's Livestock Industry in a Competitive World Market, Kumasi, October 4–7 2000.

Animal Health Australia 2010. Disease strategy: Newcastle disease (Version 3.2). Australian Veterinary Emergency Plan (AUSVETPLAN), Edition 3, Primary Industries Ministerial Council, Canberra, ACT.

ASEAN 1998. Manual of ASEAN standards for animal vaccines. Jakarta: ASEAN Secretariat, Livestock Production Series No. 2A. (http://www.aseansec.org/agr_pub/ls2.doc)

Beard, C.W. and Hanson, R.P. 1984. Newcastle disease. In: Hofstad, M.S., Barnes, H.J., Calnek, B.W., Reid, W.M. and Yoder, H.W., ed. Diseases of Poultry, 8th edn, Iowa State University Press, Ames, Iowa. pp. 452–470.

Bell, J.G. 2001. A comparison of the different vaccines available for the control of Newcastle disease in village chickens. In: Alders, R.G. and Spradbrow, P.B., ed. SADC Planning Workshop on Newcastle Disease Control in Village Chickens. Proceedings of an International Workshop, Maputo, Mozambique, 6–9 March 2000. ACIAR Proceedings No. 103:56–60.

Bensink, Z. and Spradbrow, P. 1999. Newcastle disease virus strain I-2: a prospective thermostable vaccine for use in developing countries. Veterinary Microbiology, 68:131–139.

Bermudez, A.J. 2003. Principles of disease prevention: diagnosis and control. In: Saif, Y.M., Barnes, H.J., Fadly, A.M., Glisson, J.R., McDougald, L.R., Swayne, D.E., ed. Diseases of Poultry, 11th edn, Mosby-Wolfe, Iowa State University Press, p. 41.

Bounous, D.I. and Stedman, N.L. 2000. Normal avian hematology: chicken and turkey. In: Feldman, B.F., Zinkl, J.G. and Jain, N.C., ed. Schalm's Veterinary Hematology, 5th edn, Lippincott, Williams and Wilkins. p. 1147.

Burleson, F.G., Chambers, T.M. and Wiedbrauk, D.L. 1992. Virology: a laboratory manual. Academic Press, Sydney.

Buza, J.J. and Mwamuhehe, H.A. 2001. Country report: Tanzania. In: Alders, R.G. and Spradbrow, P.B., ed. SADC Planning Workshop on Newcastle Disease Control in Village Chickens. Proceedings of an International Workshop, Maputo, Mozambique, 6–9 March 2000. ACIAR Proceedings No. 103:38–42.

Cannon, R.M. and Roe, R.T. 1982 Livestock disease surveys: a field manual for veterinarians. Australian Bureau of Animal Health, Department of Primary Industry. Australian Government Publishing Service, Canberra.

Catty, D. and Raykundalia, C. 1988. Production and quality control of polyclonal antibodies. In: Catty, D., ed. Antibodies Vol. 1. A Practical Approach. IRL Press Ltd, Oxford. p. 62.

Dias, P.T., Alders, R.G., Fringe, R. and Mata, B.V. 2001. Laboratory and field trials with thermostable live Newcastle disease vaccines in Mozambique. In: Alders, R.G. and Spradbrow, P.B., ed. SADC Planning Workshop on Newcastle Disease Control in Village Chickens. Proceedings of an International Workshop, Maputo, Mozambique, 6–9 March 2000. ACIAR Proceedings No. 103:91–96.

Doyle, T.M. 1927. A hitherto unrecorded disease of fowls due to a filter-passing virus. Journal of Comparative Pathology, 40:144–169.

EDQM, 2010. Directorate for the Quality of Medicines & HealthCare of the Council of Europe. European Pharmacopoeia, 6th edition. Strasbourg.

Freshney, R.J. 1994. Culture of animal cells: a manual of basic technique, 3rd edn, Wiley-Liss, New York.

Geering, W.A., Forman, A.J. and Nunn, M.J. 1995. Exotic diseases of animals. Australian Government Publishing Service, Canberra. pp. 422–430.

Gould, A.R., Kattenbelt, J.A., Selleck, P., Hansson, E., Della-Porta, A. and Westbury, H.A. 2001. Virulent Newcastle disease in Australia: molecular epidemiological analysis of viruses isolated prior to and during the outbreaks of 1998–2000. Virus Research, 77:51–60.

Hanson, R.P. and Brandly, C.A. 1955. Identification of vaccine strains of Newcastle disease virus. Science, 122:156–157.

Hlaing, W., Thein, M., Khin, A., Than, T.T. and Maung, T. 2000. Experiment on thermostable Newcastle disease vaccine I-2 strain. Proceedings of the Myanmar Academy of Agricultural, Forestry, Livestock and Fishery Science Annual Research Conference, Yangon, 3–5 April, 2000. pp. 50–59.

Hooper, P.T., Russell, G.M., Morrow, C.J. and Segal, I.Y. 1999. Lentogenic Newcastle disease virus and respiratory disease in Australian broiler chickens. Australian Veterinary Journal, 77:53–54.

Jawetz, E., Melnick, J.L. and Adelberg, E.A. 1962. Review of Medical Microbiology, 5th edn, Lange Medical Publications, Los Altos, California.

Johnstone, R.N. 1933. The pseudopoultry plague: the second outbreak. Journal of Agriculture of Victoria, 31:80–82.

Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture 2000. Guidelines for establishing quality systems in veterinary diagnostic testing laboratories. Report of a Joint FAO/IAEA Consultants Meeting/Workshop. Vienna International Centre, 4–8 September 2000. http://www-naweb.iaea.org/nafa/aph/public/guidelines-eng-may02.pdf

Kattenbelt, J.A., Meers, J., Gould, A.R. 2006. Genome sequence of the thermostable Newcastle disease virus (strain I-2) reveals a possible phenotypic locus. Veterinary Microbiology 114:134–41.

Kite, V.G., Boyle, D.B., Heine, H.G., Pritchard, I., Garner, M.G., East, I.J. 2007. A serological and virological survey for evidence of infection with Newcastle disease virus in Australian chicken farms. Australian Veterinary Journal 85:236–42.

Maas, R.A., Oei, H.L., Kemper, S., Koch, G. and Visser, L. 1998. The use of homologous virus in the haemagglutination-inhibition assay after vaccination with Newcastle disease virus strain La Sota or Clone 30 leads to an over estimation of protective serum antibody titres. Avian Pathology, 27:625–631.

Maclean, M.F. and Ramsay, M.J. 1999. Training course in local production of I-2 ND vaccine. FAO, Regional Office for Asia and the Pacific, Bangkok.

Mariner, J.C. 1997. The use of lyophilization in the manufacture of vaccines. In: Mowat, N. and Rweyemamu, M., ed. Vaccine Manual. The Production and Quality Control of Veterinary Vaccines for Use in Developing Countries. FAO Animal Health and Production Series No. 35. FAO, Rome, Italy. pp. 251–266.

McNulty, M.S., Adair, B.M., O'Loan, C.J. and Allan, G.M. 1988. Isolation of an antigenically unusual paramyxovirus type 1 from chickens. Avian Pathology, 17:509–513.

Mebus, C.A. 1997. The principles of good laboratory practices, including safety in vaccine production and quality control. In: Mowat, N. and Rweyemamu, M., ed. Vaccine Manual. The Production and Quality Control of Veterinary Vaccines for Use in Developing Countries. FAO Animal Health and Production Series No. 35. FAO, Rome, Italy. pp. 309–315.

Nagai, Y., Shimokata, K., Yoshida, T., Hamaguchi, M., Hnuma, M., Maeno, K., Matsumoto, T., Klenk, H.D. and Rott, R. 1979. The spread of a pathogenic and an apathogenic strain of Newcastle disease virus in the chick embryo as depending on the protease sensitivity of the virus glycoproteins. Journal of General Virology, 45:263–272.

OIE 2011a. Terrestrial Animal Health Code 2011. http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_1.1.2.htm

OIE 2011b. Newcastle disease. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011. http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/

OIE 2011c. Principles of veterinary vaccine production. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011. http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/

OIE 2011d. Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011. http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/

OIE 2011e. Tests for sterility and freedom from contamination of biological materials. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011. http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/

Osol, A., ed. 1972. Blakiston's Gould Medical Dictionary, 3rd edn, McGraw-Hill Book Company, New York.

Reed, L.J. and Muench, L.H. 1938. A simple method of estimating fifty percent endpoints. American Journal of Hygiene, 27:493–497.

Rehmani, S.F. 1995. Oral vaccination in chickens against Newcastle disease and some factors affecting vaccination in developing countries. PhD Thesis, Department of Veterinary Pathology, University of Queensland.

Rott, R. 1979. Molecular basis of infectivity and pathogenicity of myxoviruses. Archives of Virology 59:285–298.

Rovozzo, G.C. and Burke, C.N. 1973. A manual of basic virological techniques. Prentice-Hall Inc., Englewood Cliffs, N.J.

Senne, D.A. 1998. Virus propagation in embryonating eggs. In: Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E. and Reed, W.M., ed. A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th edn, American Association of Avian Pathologists, Pennsylvania. pp. 235–240.

Simmons, G.C. 1967. The isolation of Newcastle disease virus in Queensland. Australian Veterinary Journal, 43:29–31.

Soulebot, J.P., Palya, V.J., Rweyemamu, M. and Sylla, D. 1997. Quality assurance and good manufacturing practice. In: Mowat, N. and Rweyemamu, M., ed. Vaccine Manual. The Production and Quality Control of Veterinary Vaccines for Use in Developing Countries. FAO Animal Health and Production Series No. 35. FAO, Rome, Italy. pp. 297–308.

Spradbrow, P.B. 1992. Heat-stable vaccines as one approach to the control of Newcastle disease in village chickens. Newcastle Disease in Village Chickens: Control with Thermostable Oral Vaccines. Proceedings of an International Workshop, Kuala Lumpur, Malaysia, 6–10 October 1991. ACIAR Proceedings No. 39:11–14.

Spradbrow, P.B. 2001. Thermostable Newcastle disease vaccines. In: Alders, R.G. and Spradbrow, P.B., ed. SADC Planning Workshop on Newcastle Disease Control in Village Chickens. Proceedings of an International Workshop, Maputo, Mozambique, 6–9 March 2000. ACIAR Proceedings No. 103:61–66.

Spradbrow, P., Bensink, Z. and Grimes, S. 1995. Small-scale production and testing of Newcastle disease vaccine. Laboratory manual. Department of Veterinary Pathology, University of Queensland.

Spradbrow, P.B., MacKenzie, M. and Grimes, S.E. 1995. Recent isolates of Newcastle disease virus in Australia. Veterinary Microbiology, 46:21–28.

Spradbrow, P., Grimes, S., Young, M., Meers, J., Wambura, P., Bensink, Z., Lobo, Q., da Silva, A., Buza, J., Awuni, J., Msami, H. 2001. Studies on the I-2 Newcastle disease vaccine. Presentation at Coordination Meeting of ACIAR Project AS1/96/96 'Investigations into the Control of Newcastle Disease in Village Chickens in Mozambique'. Tofo, Mozambique, 7–9 November 2001.

Srinivasan, V.A., Kariath, A.P. and Bangar Raju, P.B.V.P. 1997. Bottling, labelling and packaging of vaccine. In: Mowat, N. and Rweyemamu, M., ed. Vaccine Manual. The Production and Quality Control of Veterinary Vaccines for Use in Developing Countries. FAO Animal Health and Production Series No. 35. FAO, Rome, Italy. pp. 297–308.

Terregino, C. and Capua, I. 2009. Conventional Diagnosis of Newcastle Disease Virus Infection. In: Capua, I. and Alexander, D.J., ed. Avian Influenza and Newcastle Disease, Springer-Verlag Italia. pp. 123–125.

Thayer, S.G. and Beard, C.W. 1998. Serologic procedures. In: Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E. and Reed, W.M., ed. A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th edn, American Association of Avian Pathologists, Pennsylvania. pp. 255–266.

Tu, T.D. 2001. Village chicken production in Vietnam and Newcastle disease control with thermostable vaccine. In: Alders, R.G. and Spradbrow, P.B., ed. SADC Planning Workshop on Newcastle Disease Control in Village Chickens. Proceedings of an International Workshop, Maputo, Mozambique, 6–9 March, 2000. ACIAR Proceedings No. 103:110–114.

Tu, T.D., Phuc, K.V., Dinh, N.T.K., Quoc, D.N. and Spradbrow, P.B. 1998. Vietnamese trials with a thermostable Newcastle disease vaccine (strain I-2) in experimental and village chickens. Preventive Veterinary Medicine, 34:205–214.

Vannier, P. 1997. Registration, licensing, controls and practical issues related to veterinary vaccines. In: Mowat, N. and Rweyemamu, M., ed. Vaccine Manual. The Production and Quality Control of Veterinary Vaccines for Use in Developing Countries. FAO Animal Health and Production Series No. 35. FAO, Rome, Italy. pp. 155–165.

Versteeg, J. 1985. A colour atlas of virology. Wolfe Medical Publications, London.

VICH. 2002. Testing for residual moisture. http://www.vichsec.org/pdf/03_2003/Gl26_st7.pdf

Villegas, P. 1998. Titration of biological suspensions. In: Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E. and Reed, W.M., ed. A Laboratory Manual for the Isolation and Identification of Avian Pathogens, 4th edn, American Association of Avian Pathologists, Pennsylvania. pp. 248–254.

Wambura, P.N., Kapaga, A.M. and Hyera, J.M.K. 2000. Experimental trials with a thermostable Newcastle disease virus (strain I-2) in commercial and village chickens in Tanzania. Preventive Veterinary Medicine, 43:75–83.

Wambura, P.N., Meers, J., Kattenbelt, J.A., Gould, A.R. and Spradbrow, P.B., 2007. Deduced amino acid sequences surrounding the fusion glycoprotein cleavage site and of the carboxyl-terminus of haemagglutinin-neuraminidase protein of the avirulent thermostable vaccine strain I-2 of Newcastle disease virus. Veterinary Research Communications, 31(1):105–12.

Wambura, P.N. 2009a. Vaccination of chickens using raw rice coated with novel trehalose nano-organogels containing Newcastle disease (strain I-2) vaccine. Tropical Animal Health and Production, 41:797–802.

Wambura, P.N. 2009b. Formulation of novel trehalose flakes for storage and delivery of Newcastle disease (strain I-2) vaccine to chickens. African Journal of Biotechnology, 8:6731–6734.

Wambura, P.N., 2011. Formulation of novel nano-encapsulated Newcastle disease vaccine tablets for vaccination of village chickens. Tropical Animal Health and Production, 43:165–169.

Westbury, H.A. 1979. Newcastle disease virus: some properties of Australian strains. Avian Diseases, 23:564–570.

Westbury, H.A. 1981. Newcastle disease virus in Australia. Australian Veterinary Journal, 57:292–297.

Westbury, H.A. 2001. Newcastle disease virus: an evolving pathogen? Avian Pathology, 30:5–11.

Worrall, E.E. 1997. Basic laboratory services and media preparation for vaccine production. In: Mowat, N. and Rweyemamu, M., ed. Vaccine Manual. The Production and Quality Control of Veterinary Vaccines for Use in Developing Countries. FAO Animal Health and Production Series No. 35. FAO, Rome, Italy. p. 226.

Young, M., Chikungwa, P., Ngeleja, C., Lobo, Q. and Fumo, A. 2009. Managing the risks to vaccine quality associated with the small-scale production of I-2 Newcastle disease vaccine in developing countries. In: Alders R.G., Spradbrow P.B. and Young M.P., eds. Village chickens, poverty alleviation and the sustainable control of Newcastle disease. Proceedings of an international conference held in Dar es Salaam, Tanzania, 5–7 October 2005. ACIAR Proceedings No. 131:108–112.

8.0

Glossary

Adsorption — the adhesion of a substance to an organic particle in a solution: for example, the adhesion of a virus to a cell.

Agglutination — clumping together.

Antigen — a substance that stimulates the production of antibodies when introduced into the body.

Antiseptic — a chemical agent that is applied topically to inhibit the growth of micro-organisms.

Aseptic — preventing microbial contamination of living tissues or sterile materials by excluding, removing or killing micro-organisms.

Batch — all the sealed, final containers derived from the same homogeneous bulk of vaccine and identified by a unique code number.

Batch record — essentially the written history or diary of a specific batch of vaccine. The batch record traces the manipulations of the components and raw materials once the production process begins.

Bench record — the raw data or laboratory records.

Biosecurity — all measures taken to prevent the transmission of an infectious organism from one host to another. In the case of Newcastle disease, biosecurity encompasses all measures taken to prevent the introduction of ND virus onto a farm or into a flock of chickens.

Certificate of analysis — the written specifications of the product.

Contamination — introduction of micro-organisms to sterile articles, materials or tissues.

Diluent — a liquid provided separately and used to dilute a vaccine to the proper concentration prior to administration. This is usually sterile saline or sterile water.

Dilutions — where dilutions are given for making up liquid reagents or vaccine, they are expressed as, for example, 1 to 3, meaning one part added to three parts,

- v/v — This is volume to volume (two liquids).
- w/v — This is weight to volume (solid added to a liquid).

Disinfectant — an agent that is intended to kill or remove pathogenic micro-organisms, with the exception of bacterial spores.

Dose — the amount of a biological product recommended on the label to be given to one animal of a specified age, at one time, and by a specified route of administration.

Efficacy — the specific ability of the biological product to produce the result for which it is offered, when used under the conditions recommended by the manufacturer.

Endpoint — the point in an assay where the highest dilution of the substance being tested produces a positive result. In titrating virus for infectivity, the endpoint is the highest dilution of virus that will produce a detectable effect in 50% of the inoculated hosts. It is usually determined mathematically.

Final product (lot) — all sealed final containers that have been filled from the same homogenous batch of vaccine in one working session, freeze-dried together in one continuous operation (if applicable), sealed in one working session, and identified by a unique code number.

Freedom from contamination — free of viable bacterial and fungal contaminants, and extraneous viruses which could be harmful to chickens receiving the vaccine.

Good manufacturing practice (GMP) — that part of QA that ensures that a product is manufactured in a safe, clean environment, by specified methods, under adequate supervision, with effective quality control procedures, so that the finished product meets quality standards.

Haemagglutination — the clumping together of red blood cells.

Haemagglutination inhibition — the prevention of haemagglutination.

Haemolysis — the destruction of red blood cells and release of haemoglobin.

Hazard — the ability of an agent or substance to cause harm.

In-process control — test procedures carried out during manufacture of a biological product to ensure that the product will comply with the agreed quality standards.

Isotonic — denotes a solution having the same osmotic pressure as some other solution with which it is compared, such as physiologic salt solution and the blood serum.

Log book — a book containing a permanent record of all events in the life of an item of equipment, such as maintenance, breakdowns, repairs and results of validation tests.

Master seed virus — a collection of aliquots of an organism at a specific passage level, which has been selected and permanently stored by the producer, and from which all other seed passages are derived, within permitted levels. Aliquots must be distributed into containers in a single operation, processed together in such a manner as to ensure uniformity, and stored so as to ensure stability.

Material safety data sheet (MSDS) — gives information on hazards associated with use of the chemical including fire and explosion hazard data, precautions needed when handling the chemical, how to deal with accidents and spills, and first aid treatment.

Mean (50%) embryo infectious dose (EID_{50}) — the amount of an agent capable of infecting 50% of the inoculated hosts. Usually expressed as the number of infectious units per volume. This represents the titre of the suspension.

Mean (50%) embryo lethal dose (ELD_{50}) — the amount of an agent capable of killing 50% of the inoculated hosts. Usually expressed as the number of lethal units per volume. This represents the titre of the suspension.

Passage — the natural or experimental transmission of an agent from one host to another.

Pathogenic — capable of causing disease in a susceptible host.

Potency — the relative strength of a biological product as determined by appropriate test methods (initially the potency is measured using an efficacy test in animals. Later this may be correlated with tests of antigen content, or antibody response, for routine batch potency tests).

Purity — freedom from extraneous micro-organisms and extraneous material (organic or inorganic) which could adversely affect the safety, potency or efficacy of the product, as determined by test methods appropriate to the product.

Quality — the features and characteristics of a product that influence its ability to satisfy stated or implied needs.

Quality assurance (QA) — includes all the planned actions and specifications necessary to provide adequate confidence that a product will satisfy the given requirements for safety.

Quality control — that part of GMP concerned with the taking of samples, the specifications of the product and the tests applied, together with organisation, documentation and release procedures.

Reference laboratory — a laboratory of recognised scientific and diagnostic expertise for a particular animal disease and/or testing methodology; includes capability for characterising and assigning values to reference reagents and samples.

Risk — the likelihood that the hazardous substance or agent might be harmful under specific circumstances.

Room temperature — the temperature of a comfortable working environment. Precise limits for this cannot be set, but guiding figures are 18–25°C. Where a test specifies room temperature, this should be achieved, with air conditioning if necessary; otherwise the test parameters may be affected.

Safety — freedom from properties causing undue local or systemic reactions when used as recommended or suggested by the manufacturer.

Specific-pathogen-free (SPF) — refers to animals that have been shown by the use of appropriate tests to be free from specified pathogenic micro-organisms, and also refers to eggs derived from SPF birds.

Stabiliser — a substance added to a vaccine to help to maintain a vaccine's effectiveness by keeping the antigen and other vaccine components stable during storage. Examples include lactose, sucrose, bovine serum albumin and gelatin.

Stability — ability to retain activity under specified conditions.

Standard operating procedure (SOP) — the step-by-step written instructions that describe how to perform a specific function in a process. The SOP should be concise, to the point and able to be understood by the person who is performing the procedure.

Sterility — freedom from viable contaminating micro-organisms, as demonstrated by approved and appropriate tests.

Sterilisation — the complete destruction of micro-organisms.

Thermotolerance — the term used to describe the ability of I-2 ND vaccine and the parent virus to retain a level of infectivity after exposure to heat, that is, the delayed heat degradation of the virus. For I-2 ND vaccine it is defined by the length of time the vaccine will retain an infectivity titre sufficient to induce a protective immune response, at a particular temperature. The terms 'heat resistant', 'delayed heat degradation' and thermostable may also be encountered. In our experience, the use of the term 'thermostable' creates unrealistic expectations of a vaccine's properties. Therefore, we prefer to use the term 'thermotolerant'.

Titre — an expression of the strength of a solution: for example, the concentration of infectious virus present in a sample or a measure of the concentration of a specific antibody in serum.

Validation — proof that the procedure, process, equipment, material, activity or system leads to the results expected.

Virulent — causing severe disease.

Working seed virus — a collection of aliquots of a virus derived from the master seed virus and from which vaccine is produced.

9.0

Appendixes

Appendix 1.1
Phosphate-buffered saline (calcium- and magnesium-free)

Equipment and materials

Disodium hydrogen orthophosphate (anhydrous) — Na_2HPO_4 — Mol. wt 142
or — $Na_2HPO_4.2H_2O$ — Mol. wt 178
or — $Na_2HPO_4.12H_2O$ — Mol. wt 358
Potassium dihydrogen orthophosphate — KH_2PO_4 — Mol. wt 136.1
Sodium chloride — NaCl — Mol. wt 58.44
Potassium chloride — KCl — Mol. wt 74.56
Distilled water
Balance
Hot plate magnetic stirrer
pH meter
Conical flask (1 L or 5 L)
Bottles (100 mL, 250 mL, 500 mL)
Measuring cylinder (1 L)

Procedure

1. Collect distilled water directly from the still on the day of preparation.
2. Weigh all salts according to the formula presented in the table below and place in a flask.

Starting materials	g/L	g/5 L
Disodium hydrogen orthophosphate (anhydrous)	0.92	4.6
or Disodium hydrogen orthophosphate ($2H_20$)	1.15	5.75
or Disodium hydrogen orthophosphate ($12H_20$)	2.32	11.6
Potassium dihydrogen orthophosphate	0.2	1.0
Potassium chloride	0.2	1.0
Sodium chloride	8.0	40.0
Distilled water	Make up to 1 L	Make up to 5 L

3. Put a magnetic stirring bar into the flask.
4. Add 800 mL (or 4.8 L) distilled water to the salts (use 1 L cylinder to measure volume of water).
5. Place the flask on hot plate magnetic stirrer and allow the salts to dissolve.
6. After all the salts have dissolved, allow to cool to room temperature (25°C) and adjust volume of the solution to the exact final volume required.
7. Calibrate the pH meter.
8. Adjust the pH of PBS to pH 7.2 (use concentrated HCl to lower the pH).
9. Dispense PBS into bottles and autoclave at 121°C for 15 minutes.
10. Label all bottles clearly, writing sterility status (sterile/not sterile) and date of preparation on the label.
11. Store at room temperature.

Appendix 1.2
10% Buffered neutral formalin

Safety precautions

Formaldehyde is toxic by inhalation and irritating to the eye, skin and respiratory system. Use gloves when handling formalin.

Prepare the solution in a fume hood.

Equipment and materials

Disodium hydrogen phosphate — Na_2HPO_4 (anhydrous) — Mol. wt 142
Potassium dihydrogen phosphate — KH_2PO_4 — Mol. wt 136
Formalin (40% formaldehyde solution)
Distilled water
Balance
Hot plate magnetic stirrer
pH meter
Conical flask (1 L or 5 L)
Bottles (100 mL, 250 mL, 500 mL)
Measuring cylinder (1 L)

Procedure

1. Collect distilled water directly from the still on the day of preparation.
2. Weigh all salts according to the formula presented in the table below and place in a flask.

Starting materials	g/L	g/5 L
Disodium hydrogen orthophosphate (Na_2HPO_4) (anhydrous)	6.55	32.75
Potassium dihydrogen phosphate (KH_2PO_4)	4.0	20.0
Distilled water	Make up to 1 L	Make up to 5 L

3. Add 100 mL (or 500 mL) formalin.
4. Make up to 1 L (or 5 L) with distilled water.
5. Store at room temperature.

NOTE:

- This solution is used as a general tissue fixative.
- The volume of fixative used should be at least ten times that of the tissues.
- The thickness of tissue slices should be no greater than 5 mm.
- 10% formalin can also be prepared by mixing formalin with tap water (but formalin pigment will be seen in tissues).

Appendix 1.3
3.5% Iodine solution

Safety precautions

Iodine is irritating to the eyes, skin and mucous membranes.

Wear gloves when handling and mixing chemicals.

Wear safety glasses or face shield when handing and mixing chemicals, or prepare the solution in a fume hood.

Equipment and materials

Iodine — I_2 — Mol. wt 253.81
Ethyl alcohol (95%)
Water

Procedure

1. Dissolve 3.5 g iodine in 30 mL water.
2. Dilute to 100 mL with 95% ethyl alcohol.

or

Equipment and materials

Iodine — I_2 — Mol. wt 253.81
Sodium iodide — NaI — Mol. wt 149.92

Procedure

1. Dissolve 3.5 g iodine and 1.5 g sodium iodide in 30 mL water.
2. Dilute to 100 mL with 95% ethyl alcohol.

Appendix 1.4
50% Glycerol phosphate buffer

Equipment and materials

Disodium hydrogen phosphate	— $Na_2HPO_4.12H_2O$	— Mol. wt 358
Potassium dihydrogen orthophosphate	— KH_2PO_4	— Mol. wt 136.09
Glycerol AR	— $C_3H_8O_3$	— Mol. wt 92.1

Distilled water
Balance
Magnetic stirrer
pH meter
Conical flask (1 L or 5 L)
Bottles (100 mL, 250 mL, 500 mL)
Measuring cylinder (1 L)

Procedure

0.04 M Phosphate Buffer (pH 7.6)

1. Prepare the following two solutions:

   Solution A

   — Weigh 7.13 g disodium hydrogen phosphate into a flask.

   — Add 1 L distilled water.

   Solution B

   — Weigh 5.45 g potassium dihydrogen phosphate into a flask.

   — Add 1 L distilled water.

2. Prepare 0.04 M phosphate buffer (pH 7.6)

   — Mix six parts of solution A (for example, 600 mL) with one part of solution B (for example, 100 mL) to give a buffer of pH 7.6.

   — Check pH using a pH meter, and adjust if necessary.

   — Sterilise by autoclaving and recheck the pH.

Glycerol phosphate buffer

1. Add equal parts of glycerol AR and 0.04 M phosphate buffer.
2. Sterilise by autoclaving.
3. Check and adjust the pH.
4. Store at room temperature.

NOTE:

- 50% glycerol can also be prepared using PBS or distilled water.
- The majority of pathogenic bacteria do not survive 5–6 days in 50% glycerol (Jawetz, Melnick and Adelberg 1962).

Appendix 1.5
70% Alcohol

Equipment and materials

Absolute ethanol or 95% ethanol
Distilled water
Measuring cylinder (100 mL or 1 L)

Procedure

1. Mix 70 mL of absolute ethanol (100%) with 30 mL distilled water.

or

Mix 70 mL 95% ethanol with 25 mL distilled water.

Appendix 1.6
Alsever's solution

Equipment and materials

Dextrose (D + Glucose)	— $C_6H_{12}O_6$	— Mol. wt 180.2
Sodium citrate	— $C_6H_5Na_3O_7.2H_2O$	— Mol. wt 294.1
Sodium chloride	— NaCl	— Mol. wt 58.44
Citric acid	— $C_6H_8O_7$	— Mol. wt 192.1

Distilled water
Balance
Magnetic stirrer
pH meter
Conical flask (1 L or 5 L)
Bottles (100 mL, 250 mL, 500 mL)
Measuring cylinder (1 L)

Procedure

1. Collect distilled water directly from the still on the day of preparation.
2. Weigh all salts according to the formula presented in the table below.

Starting materials	g/100 mL	g/L
Dextrose	2.05	20.5
Sodium citrate	0.8	8.0
Sodium chloride	0.42	4.2
Citric acid	0.055	0.55
Distilled water	Make up to 100 mL	Make up to 1 L

3. Transfer to a 1 L measuring cylinder and make up to required volume with distilled water.
4. Adjust the pH to 6.1 using a newly prepared solution of 10% citric acid.
5. Dispense into bottles.
6. Sterilise by autoclaving at 121°C for 15 minutes. Use slow exhaust.
7. Store at 4°C. The solution remains stable for long periods of time.

Appendix 1.7
Acid-citrate-dextrose (ACD) solution

Equipment and materials

Sodium citrate — $C_6H_5Na_3O_7.2H_2O$ — Mol. wt 294.1
Citric acid — $C_6H_8O_7$ — Mol. wt 192.1
Dextrose (D(+)-Glucose) — $C_6H_{12}O_6$ — Mol. wt 180.2
Distilled water
Balance
Magnetic stirrer
pH meter
Conical flask (1 L or 5 L)
Bottles (100 mL, 250 mL, 500 mL)
Measuring cylinder (1 L)

Procedure

1. Collect distilled water directly from the still on the day of preparation.
2. Weigh all salts into a glass beaker according to the formula presented in the table below.

Starting materials	g/500 mL	g/1 L
Citric acid	4.0	8.0
Sodium citrate	11.26	22.52
Dextrose	11.0	22.0
Distilled water	500 mL	1 L

3. Make up to 500 mL (or 1 L) with distilled water.
4. Dispense into bottles.
5. Sterilise by autoclaving at 121°C for 15 minutes.
6. Store at 4°C.

Appendix 1.8
Dextrose-gelatin-veronal (DGV) solution

Equipment and materials

Barbitone (diethylbarbituric acid, veronal) — $C_8H_{12}N_2O_3$ — Mol. wt 184.2
Gelatin
Barbitone sodium — $C_8H_{11}N_2O_3Na$ — Mol. wt 206.2
Calcium chloride dihydrate — $CaCl_2.2H_2O$ — Mol. wt 147
or calcium chloride (anhydrous) — $CaCl_2$ — Mol. wt 111
Magnesium sulphate — $MgSO_4.7H_2O$ — Mol. wt 246.5
Sodium chloride — NaCl — Mol. wt 58.44
Dextrose (D(+)-Glucose) — $C_6H_{12}O_6$ — Mol. wt 180.2
Distilled water
Balance
Hot plate magnetic stirrer
Beakers (1 L)
Measuring cylinder (1 L)
100 mL bottles

Procedure

For 1 L:

1. Collect distilled water directly from the still on the day of preparation.
2. Weigh barbitone and gelatin into a beaker according to the formula presented in the table below.

Starting materials	g/1 L	g/2 L
Barbitone (diethylbarbituric acid)	0.58	1.16
Gelatin	0.60	1.20
Barbitone sodium	0.30	0.60
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.026	0.052
or Calcium chloride anhydrous ($CaCl_2$)	0.02	0.04
Magnesium sulphate ($MgSO_4.7H_2O$)	0.12	0.24
Sodium chloride (NaCl)	8.50	17.0
Dextrose	10.0	20.0
Deionised distilled water	1 L	2 L

3. Dissolve in 250 mL (or 500 mL) deionised distilled water on a hot plate magnetic stirrer.
4. Weigh remaining reagents into a beaker and dissolve in remainder of water.
5. Add dissolved barbitone to remaining reagents.
6. Make up to 1 L (or 2 L).
7. Measure pH and adjust to 7.0–7.6.
8. Pour into 100 mL bottles.
9. Autoclave at 121°C for 15 minutes.
10. Store at 4°C.

NOTE:

The supply and use of Veronal (barbitone, diethylbarbituric acid), one of the components of DGV, may be restricted by government regulation.

Appendix 2
General list of laboratory glassware and consumables

Glassware

Pipettes — 1 mL, 5 mL, 10 mL
Glass Pasteur pipettes
Conical flasks — 50 mL, 100 mL, 500 mL, 1 L, 2 L
Beakers — a range of sizes
Tubes for serial dilutions — to 10 mL
Measuring cylinders — a range of sizes
Glass bottles with screw caps 20–30 mL, 5–10 mL
Large glass bottles with screw caps 200 mL, 500 mL, 1 L, 2 L

Consumables

Syringes — 1 mL, 2.5 mL, 5 mL, 10 mL, 20 mL
Needles — 23 G × 32 mm, 25 G × 16 mm
Tips for micropipettor
Eppendorf tubes
Cryotubes
96-well V-bottomed microtitre plates
Plastic centrifuge tubes — 10 mL, 50 mL
Plastic centrifuge bottles — 500 mL, 1 L, 2 L
Alcohol
Adhesive tape, wax or Colloidion (to seal inoculation site in eggs)
Penicillin, Streptomycin or other antibiotics, depending on availability
General laboratory chemicals (see Appendixes 1.1 to 1.8)
Labels
Vials, stoppers, aluminium caps for freeze-dried vaccine
Small bottles or disposable pipettes for 'wet' vaccine

Equipment

Egg candling lamp
Egg incubator
Bunsen burner
Electronic balance
Magnetic stirrer
Centrifuge
Vaccine dispenser
Shell cutter
Shell punch
Test tube racks
Single channel and multichannel pipettors

Appendix 3
Registration of vaccine

Every product sold to treat or prevent disease in animals should be registered with the national authority responsible for the regulation of use of such products. Registration of locally manufactured or imported products ensures that they meet accepted standards of quality, safety and efficacy, thereby protecting livestock and helping to maintain food quality and hygiene.

Vaccine producers or importers should work together with the registration authority to encourage the formulation of regulations and standards which are realistic given the human and financial resources available, but which will lead to improvements in the standards of products manufactured and/or used.

The registration authority will define the conditions for registration and should give directions on how to apply for registration of the product, the documentation required and other information including:

- the conditions of registration
- how to file an application for registration
- what is needed, for example:
  - a licence of manufacture
  - premises inspection
  - information dossier
  - data files and records of all stages of manufacture (including testing)
  - specifications of the vaccine
  - data for shelf-life validation
  - standard operating procedures.

The **information dossier** should contain information to support the application for registration — for example, expert reports, analytical details, the description and results of safety tests, efficacy trials and all documentation to support the registration (Vannier 1997). An outline or template for the information dossier should be available from the registration authority.

A standard operating procedure (SOP) should be written for each specific task in the vaccine production process. All tasks, such as calibration or maintenance of a micropipettor, validation of a process such as autoclaving, cleaning of an egg incubator or placement and monitoring of insect or pest traps in the vaccine production chicken house must be documented in an SOP. The SOP must be clear, concise, to the point and written in language that can be readily understood.

The key elements of an SOP are:

- the cover sheet showing the:
  - title
  - procedure number
  - department
  - page number
  - date on which SOP becomes effective
  - signature of the persons responsible for SOP
  - name of the person responsible for writing the SOP.

- the objective, which explains the main purpose of the procedure. It defines whether the purpose is to outline the method of operation, maintenance etc.
- the scope of the SOP, which gives the limitations of the procedure
- the position or title and roles of the persons responsible for carrying out the procedure
- the procedure, giving step-by-step directions written in words that the person performing the procedure or task will understand
- the dates for review and retention. SOPs should be reviewed at least once each year.

The preparation of documents such as SOPs, job descriptions and a laboratory Quality Manual is discussed in *Guidelines for establishing quality systems in veterinary diagnostic testing laboratories* (Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture 2000).

Appendix 4
Using a multichannel pipettor

1. Select a pipettor of the appropriate volume range.
2. Set the volume to be delivered, according to the manufacturer's instructions.
3. Fit new disposable tips. Ensure that the tip is the correct type for the pipettor. To ensure correct fit, press the tips on firmly, using a slight twisting motion.
4. **Never use a pipettor without tips.**
5. Hold the pipettor vertically; press down the plunger or button until the first stop.
6. Place the end of the tips in the liquid; release pressure slowly and evenly, keeping the thumb on the button or plunger. Watch as the liquid is drawn up into the tips. Confirm that no air bubbles are present in the tips.
7. Withdraw the tips from the liquid and confirm that all the tips contain the same level of liquid.
8. Hold the end of the tip against the wall of the receiving vessel at a slight angle to deliver the liquid. Press the plunger down slowly and smoothly to the first stop. Allow the residual liquid to run down to the tip and press the plunger down to the final stop.
9. Eject the used tips into an appropriate container by pressing the tip ejector button. Always eject the used tips before putting the pipettor on the bench since any residual fluid may run back into the barrel of the pipettor.

   — Regular cleaning and calibration of the pipettor is essential. Follow the manufacturer's instructions provided with the instrument.

   — For calibration, dispense volumes of distilled water into a pre-weighed beaker or weighing boat, and weigh using an analytical balance. Assume that 1 mg = 1 µL.

Appendix 5
How to convert r.p.m. to relative centrifugal force (RCF)

Centrifuge speed (the speed of rotation of the rotor) is measured in revolutions per minute (r.p.m.). However, a more correct measure of centrifuge function is relative centrifugal force (RCF), the force exerted on the material being centrifuged. RCF depends on the speed of the rotor and the radius of rotation, and is expressed as × *g*, a multiple of the acceleration due to gravity.

To convert RCF to r.p.m.

1. Measure the radius of the centrifuge rotor, including the buckets when in the horizontal position (in centimetres).
2. Using the ruler and the nomogram (Figure 19), draw an imaginary line connecting the radius and RCF (*g*).
3. Read off the r.p.m. where the ruler crosses the r.p.m. line.

To convert r.p.m. to RCF

4. Measure the radius of the centrifuge rotor (in centimetres).
5. Using the ruler and the nomogram (Figure 19), draw an imaginary line connecting the radius and r.p.m.
6. Read off the RCF (*g*) where the ruler crosses the *g* line.

Example

The protocol requires that a sample is centrifuged at RCF of 500 × *g*. The radius of centrifuge rotor you are using is 12 cm. Using the nomogram, it can be seen that your centrifuge will have to be set at 1900 r.p.m.

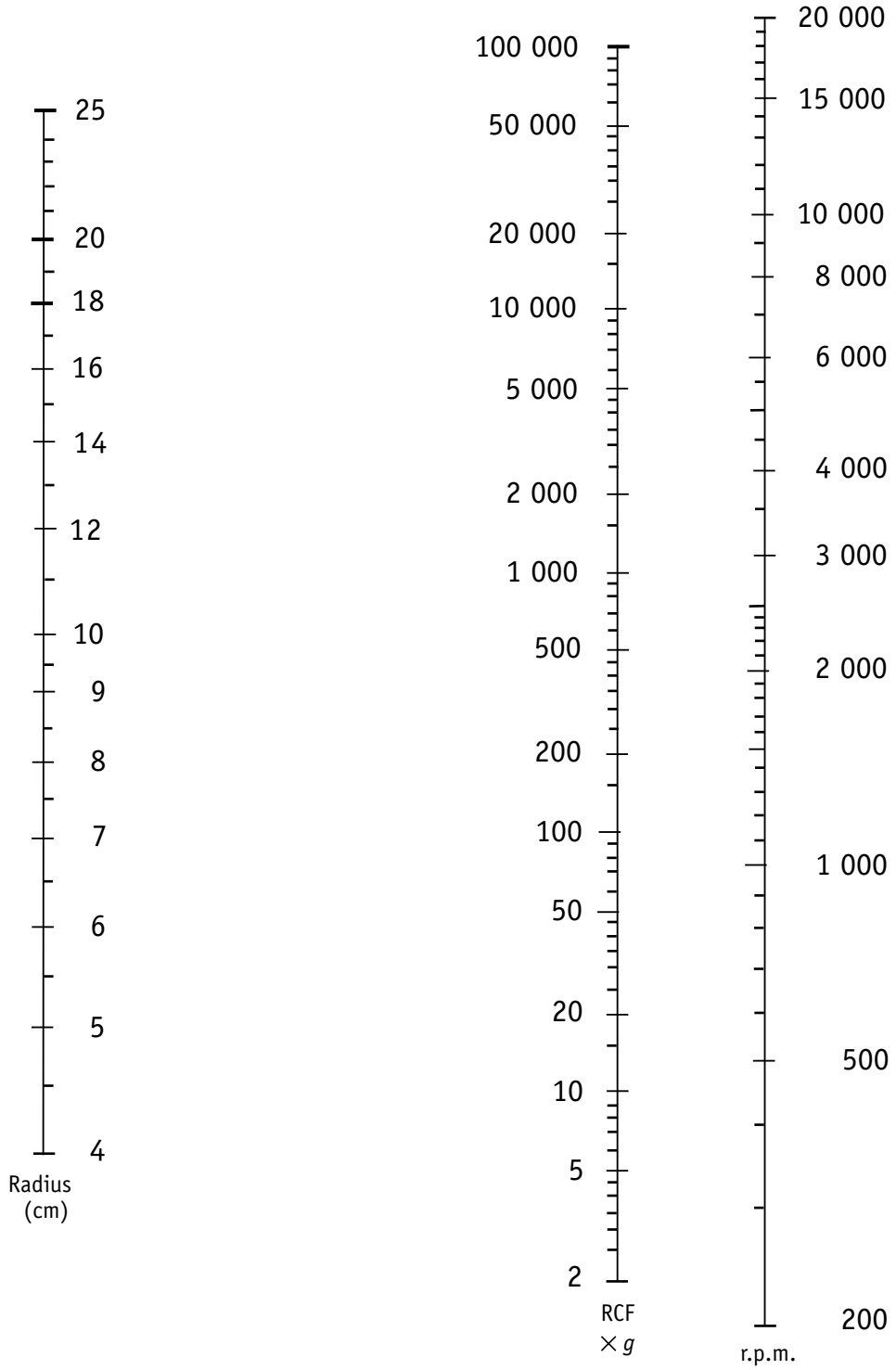


Figure 19: *The Nomogram: an easy way to convert maximum relative centrifugal force (RCF) to r.p.m. or r.p.m. to RCF.*

Appendix 6
The use of antibiotics in vaccine production

The use of antibiotics as a safeguard against contamination during vaccine production should be avoided wherever possible. Antibiotics are expensive, sometimes difficult to obtain, and add to the cost of production of the vaccine. The vaccine producer should also encourage more responsible use of antibiotics and opt instead for improved aseptic technique. In addition, many registration authorities do not allow the use of antibiotics as preservatives in vaccines, only approving the addition of antibiotics to vaccine diluent to protect from 'in use' contamination.

Table 5 lists the antibiotics permitted in pharmaceuticals by US standards (Mebus 1997) together with their spectrum of use.

Table 5: *Antibiotics permitted in pharmaceuticals (Mebus 1997) and their spectrum of activity*

Antibiotic name	Permitted concentration per mL	Gram + bacteria	Gram – bacteria	Yeasts	Molds	Mycoplasma
Amphotericin B	2.4 µg			■	■	
Nystatin	30.0 U			■	■	
Tetracyclines	30.0 µg	■	■			
Penicillin	30.0 U	■				
Streptomycin	30.0 µg	■	■			
Polymixin B	30.0 µg		■			
Neomycin	30.0 µg	■	■			
Gentamycin	30.0 µg	■	■			■

Combinations of antibiotics permitted by US regulations are:

- penicillin and streptomycin
- amphotericin B and any one of the other antibiotics shown in Table 5
- nystatin and any one of the other antibiotics shown in Table 5
- amphotericin B (or nystatin) with a combination of penicillin and streptomycin
- amphotericin B (or nystatin) with a combination of polymixin B and neomycin

Appendix 7
Calculation of titre using the method of Spearman-Kärber

Some workers prefer to use the method of Spearman-Kärber to calculate the titre of virus (Villegas 1998). Generally, this method does not involve a great number of calculations, and the results are reported to be as accurate as those obtained using the Reed and Muench formula. However, this method can only be used where the results of the dilutions tested cover the full 0–100% infection range.

A. Where the number of eggs at each dilution is constant, the titre of the virus suspension (EID_{50}) is calculated using the following formula:

$$EID_{50} = x + \frac{1}{2}d - \frac{d\Sigma r}{n}$$

where x = the highest dilution at which all eggs were uninfected (expressed as the reciprocal, or positive value)

d = the dilution factor (where tenfold dilutions have been used, the dilution factor is 1)

Σr = the total number of uninfected eggs (in the dilution range 0–100% infected)

n = the number of eggs at each dilution

Since the volume of the inoculum used was 0.1 mL, the titre is expressed as EID_{50} per 0.1 mL.

Using the data presented in Section 4.4.1:

Dilution	HA results	No. of infected eggs	No. of uninfected eggs
10^{-1}	+ + + + +	5	
10^{-2}	+ + + + +	5	
10^{-3}	+ + + + +	5	
10^{-4}	+ + + + +	5	
10^{-5}	+ + + + +	5	0
10^{-6}	+ + + + –	4	1
10^{-7}	+ + + – –	3	2
10^{-8}	+ + – – –	2	3
10^{-9}	– – – – –	0	5
10^{-10}	– – – – –	0	

\+ Infected.
– Uninfected.

1. Fill in the number of infected eggs and the number of uninfected eggs (columns 3 and 4) for the dilution range 0–100% infected.

2. Using the Spearman-Kärber method:

$$EID_{50} = x + \frac{1}{2}d - \frac{d\Sigma r}{n}$$

$x = 9$

$d = 1$

$\Sigma r = 5 + 3 + 2 + 1 + 0$

$n = 5$

Therefore: $EID_{50} = 9 + \frac{1}{2} \times 1 - \frac{1(5 + 3 + 2 + 1 + 0)}{5}$

$= 9 + 0.5 - \frac{11}{5}$

$= 9.5 - 2.2$

$= 7.3$

Therefore, titre of the virus suspension = $10^{7.3}$ EID_{50} per 0.1 mL

B. Where the number of eggs at each dilution is not constant, for instance if non-specific death of embryos has occurred, the calculations become more complex (Allan et al. 1978). The EID_{50} is calculated using the following equation:

$$EID_{50} = x + \frac{1}{2}d - d\Sigma p$$

where x = the highest dilution at which all eggs were uninfected (expressed as the reciprocal, or positive value)

d = the dilution factor (where tenfold dilutions have been used, the dilution factor is 1)

Σp = the sum of the proportions of uninfected eggs (in the dilution range 0 to 100% infected)

Using the data presented in Section 4.4.2, Exercise 5:

Dilution	HA results	No. of infected eggs	No. of uninfected eggs	Proportion of uninfected eggs
10^{-1}	+ + + + +	5	0	
10^{-2}	+ + + + 0	4	0	
10^{-3}	+ + + 0 0	3	0	0
10^{-4}	+ + + + –	4	1	1/5 = 0.2
10^{-5}	+ + + 0 0	3	0	0
10^{-6}	+ + – – 0	2	2	2/4 = 0.5
10^{-7}	+ + – – 0	2	2	2/4 = 0.5
10^{-8}	+ – – – –	1	4	4/5 = 0.8
10^{-9}	– – – – –	0	5	1
10^{-10}	– – – – –	0	5	

1. Fill in the number of infected eggs and the number of uninfected eggs (columns 3 and 4) for the dilution range 0–100% infected.
2. Calculate the proportion of uninfected eggs (column 5).

3. Using the Spearman-Kärber method:

$$EID_{50} = x + \frac{1}{2}d - d\Sigma p$$

$x = 9$

$d = 1$

$\Sigma p = (1 + 0.8 + 0.5 + 0.5 + 0 + 0.2 + 0) = 3$

$$EID_{50} = 9 + \frac{1}{2} - 1 \times 3$$

$= 9 + 0.5 - 3$

$= 6.5$ per 0.1 mL of virus suspension

Therefore, the titre of the virus suspension = $10^{6.5}$ EID_{50} per 0.1 mL

Appendix 8
How to prepare serial dilutions

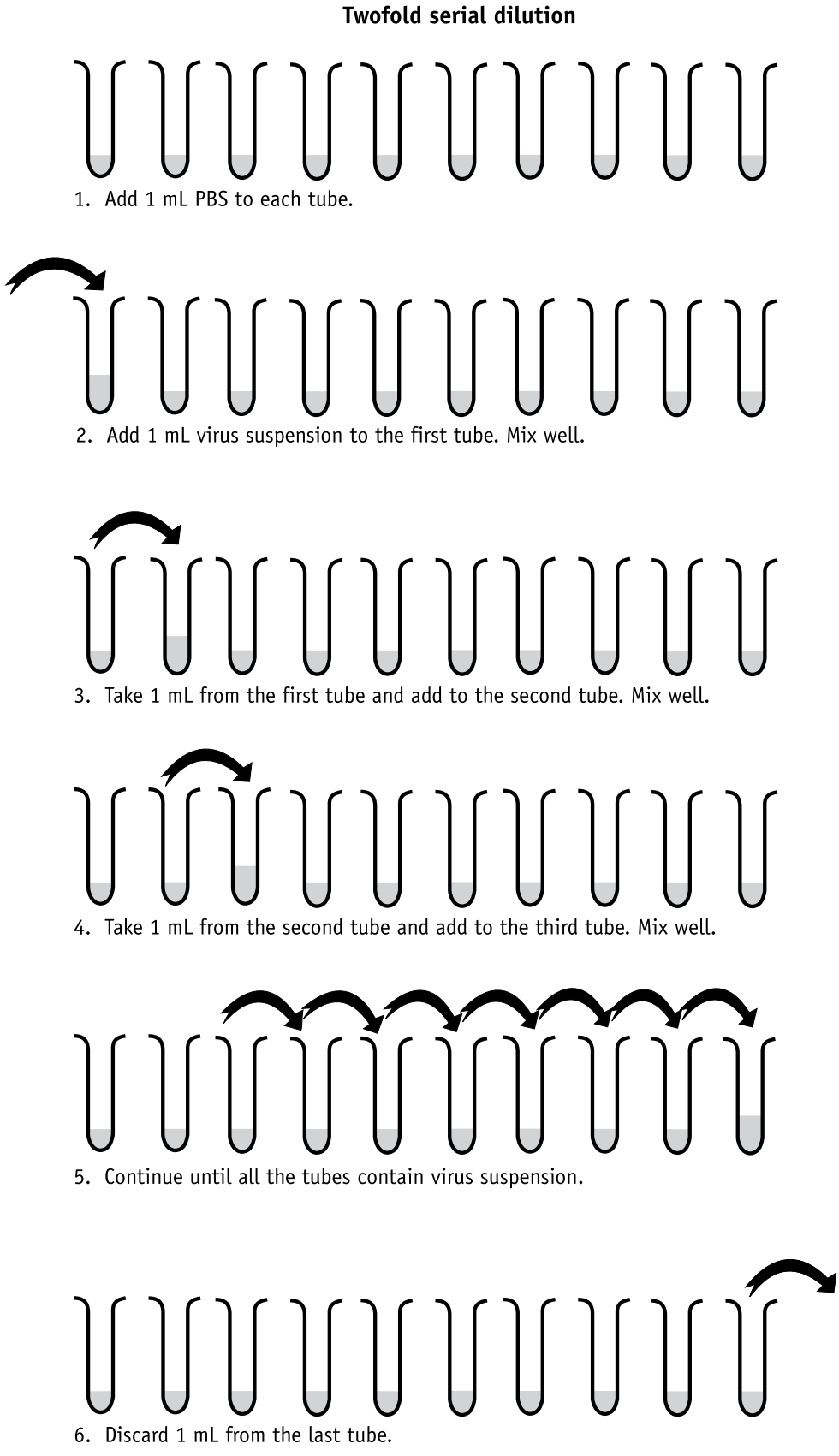


Figure 20: *Twofold serial dilutions.*

Tenfold serial dilution

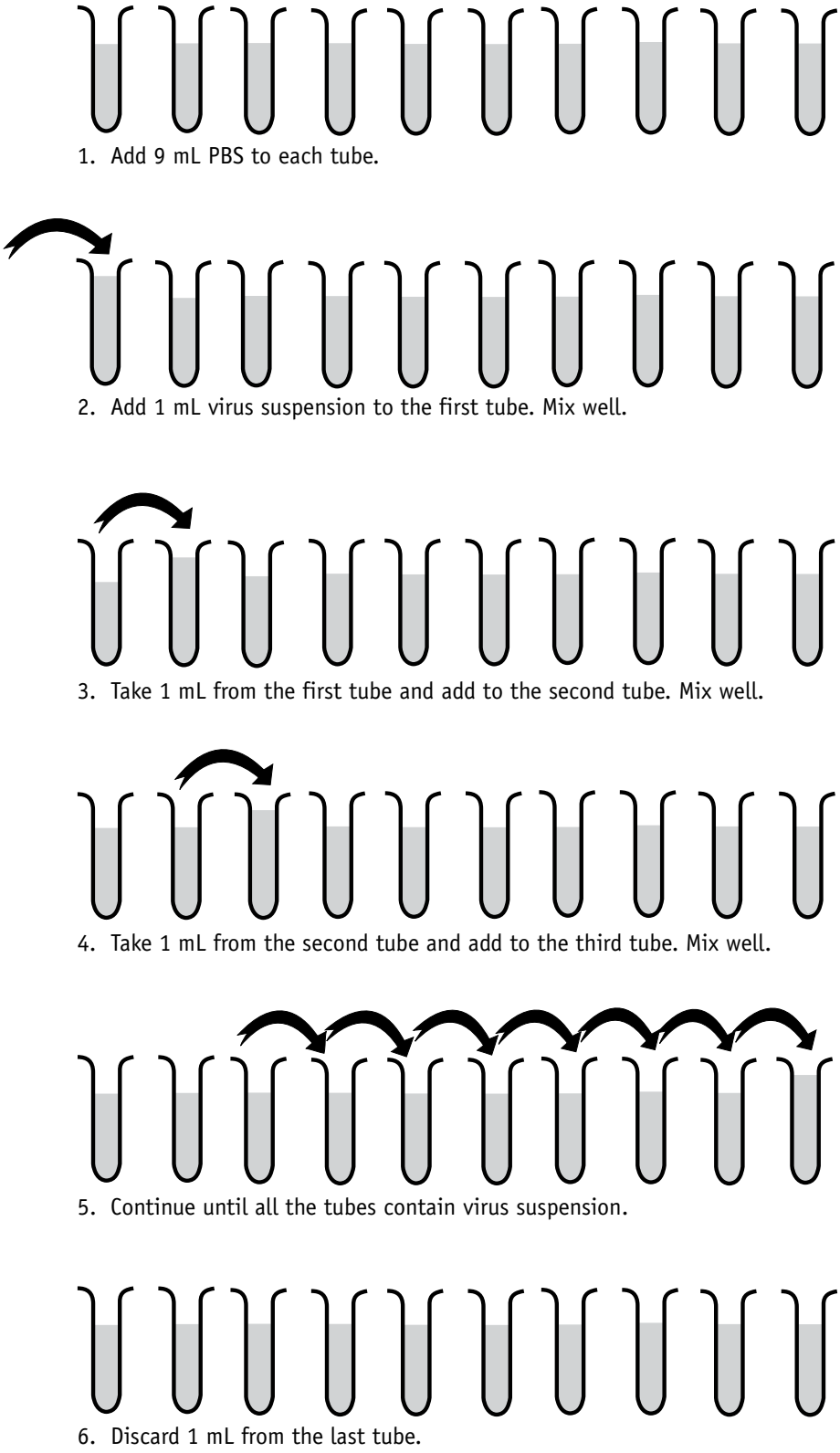


Figure 21: *Tenfold serial dilutions.*

Appendix 9
Basic instructions on the administration of live, thermotolerant Newcastle disease vaccine

A. Freeze-dried vaccine

By eye-drop

- The vaccine will protect against Newcastle disease **only** (use the local name for ND when appropriate).
- Do not vaccinate sick chickens.
- Chickens may be eaten immediately after vaccination.
- The vaccine will not harm chickens, affect growth or egg-laying.
- The vaccine will not cause problems in people if it is spilled on the skin.
- It takes **7 to 14 days** for a chicken to develop adequate protection against Newcastle disease after vaccination.
- Chickens should be revaccinated **every 4 months**[a] as their level of protection will start to fall after this period.
- A dose (one eye-drop) is **the same for chickens of all ages** (from day old to adult) and for males and females.
- If all of the first drop did not enter the eye, apply a second drop.
- Use the eye-dropper supplied by .....[b] and confirm the volume of diluent or potable water required to dilute the vaccine the day before vaccination. A guide to determining the volume is given on the back of this sheet[c].

Preparing water suitable for dilution of the vaccine

If .....[b] diluent is not available:

— Boil local drinking water and leave to cool in a covered container,

— Do not use metal containers to store boiled water,

— Do not use treated tap water because the chlorine will destroy the vaccine (if no other water is available, let the tap water stand overnight to allow the chlorine to go away).

Storage and durability of vaccine

— This vaccine is thermotolerant but you still need to treat it carefully!

— DO NOT FREEZE.

— In the refrigerator at 4°C it will last until the expiry date given on the label, if not diluted.

— Outside the refrigerator if stored in a cool, dark place it will last for .....[d] if not diluted.

— Transport in the field using a cool box and ice pack if available or wrap the vial in a damp cloth and carry it in a covered, open-weave basket (to keep it cool and away from sunlight).

After diluting the vaccine, use within two days according to the following guide:

— Day 1 ⇛ 1 eye-drop per chicken (i.e. first day of vaccination campaign)

— Day 2 ⇛ 2 eye-drops per chicken

— Day 3 ⇛ throw away.

For further information contact: .....[b]

---

a Insert the interval appropriate to local recommendations.
b Insert name of vaccine manufacturer.
c See Appendix 10.
d Insert interval determined during stability testing of vaccine.

By cooked white rice*

In some places, it may be very difficult to catch chickens for vaccination and the vaccinators may wish to administer vaccine on feed. The use of feed carriers to administer I-2 ND vaccine is not as effective as eye-drop vaccination.

- The vaccine will protect against Newcastle disease **only** (use the local name for ND when appropriate).
- **Do not vaccinate sick chickens.**
- Chickens may be eaten immediately after vaccination.
- The vaccine will not harm chickens, affect growth or egg-laying.
- The vaccine will not cause problems in people if spilled on the skin.
- A dose is **the same for chickens of all ages** (from day old to adult) and for males and females.
- One dose is diluted in 1 mL of clean, unchlorinated water and mixed with cool, cooked white rice immediately prior to administration. Allow 20 g of cooked rice (7–10 g of raw rice that is then cooked) per bird. A booster vaccination after 2 weeks is essential.
- This dose is best administered in the morning when chickens are hungry and more likely to eat a full dose.
- It takes **7 to 14 days** for a chicken to develop adequate protection against Newcastle disease after the booster vaccination.
- Chickens should be revaccinated **every 2 months**[e] as their level of protection will start to fall after this period.

Preparing water suitable for dilution of the vaccine

— Boil local drinking water and leave to cool in a covered container.

— Do not use metal containers to store boiled water.

— Do not use treated tap water because the chlorine will destroy the vaccine (if no other water is available, let the tap water stand overnight to allow the chlorine to go away).

Storage and durability of vaccine

— This vaccine is thermotolerant but you still need to treat it carefully!

— DO NOT FREEZE.

— In the refrigerator at 4°C it will last until the expiry date given on the label, if not diluted.

— Outside the refrigerator if stored in a cool, dark place it will last for .....[f] if not diluted.

— Transport in the field using a cool box and ice pack if available or wrap the vial in a damp cloth and carry it in a covered, open-weave basket (to keep it cool and away from sunlight).

For further information contact: .....[g]

*If vaccinators wish to use an alternative feed carrier, only carriers tested and recommended by the vaccine manufacturer or distributor should be used. The vaccine manufacturer or distributor must test feeds and grains prepared in various ways for virucidal activity (Rehmani 1995). To ensure an adequate immune response to vaccination it is essential that the chickens consume the entire dose of vaccine and that booster vaccinations are given. Various carriers including boiled white rice, oiled white rice, paddy rice and maize products have been used.

e Insert the interval appropriate to local recommendations.
f Insert the interval determined during stability testing of vaccine.
g Insert name of vaccine manufacturer.

B. 'Wet' vaccine

- The vaccine will protect against Newcastle disease **only** (use the local name for ND when appropriate).
- **Do not vaccinate sick chickens.**
- Chickens may be eaten immediately after vaccination.
- The vaccine will not harm chickens, affect growth or egg-laying.
- The vaccine will not cause problems in people if it is spilled on the skin.
- It takes **7 to 14 days** for a chicken to develop adequate protection against Newcastle disease after vaccination.
- Chickens should be revaccinated **every 4 months**[h] as their level of protection will start to fall after this period.
- A dose (one eye-drop) is **the same for chickens of all ages** (from day old to adult) and for males and females.
- If all of the first drop did not enter the eye, apply a second drop.

Storage and durability of vaccine

— This vaccine is thermotolerant but you still need to treat it carefully!

— DO NOT FREEZE.

— In the refrigerator at 4°C it will last until the expiry date given on the label.

— Transport in the field using a cool box and ice pack if available or wrap the vial in a damp cloth and carry it in a covered, open-weave basket (to keep it cool and away from sunlight).

Use within two days according to the following guide:

— Day 1 ⇛ 1 eye-drop per chicken (i.e. first day of vaccination campaign)

— Day 2 ⇛ 2 eye-drops per chicken

— Day 3 ⇛ throw away.

For further information contact: .....[i]

h Insert the interval appropriate to local recommendations.
i Insert name of vaccine manufacturer.

Appendix 10
How to confirm the volume of water required to dilute freeze-dried vaccine

If a dropper is not supplied with the freeze-dried vaccine, you will need to calculate how much diluent to add to reconstitute the vaccine. To do this:

1. Select an eye-dropper.
2. Aspirate water into the dropper.
3. Remove the plunger from a 2 mL syringe and hold the syringe vertically with the tip down. Close the tip with a finger or thumb.
4. Hold the dropper vertically, squeeze the bulb gently and allow the drops to fall into the syringe.
5. Count the number of drops required for the water level to reach the 1 mL mark on the syringe (you may need to refill the dropper).
6. Write down the number of drops required to form 1 mL. Repeat at least two more times.
7. Use the following formula to calculate the volume of diluent required to dilute the number of doses of vaccine in the vial.

$$\text{Volume of diluent (mL)} = \frac{\text{No. of doses of vaccine per vial}}{\text{No. of drops formed per mL}}$$

Example

An eye-dropper delivers 50 drops of water in 1 mL. How much diluent should be added to a vial containing 250 doses of ND vaccine?

$$\text{Volume of diluent (mL)} = \frac{\text{250 doses per vial}}{\text{50 drops per mL}}$$

$$= \text{5 mL}$$

Appendix 11
Answers to the exercises

3.7.2

1. (a) Titre of wet vaccine per dose:

$1\ mL = 0.1\ mL \times 10$

Titre of vaccine per mL $= 10^9\ EID_{50} \times 10$

$= 10^9\ EID_{50} \times 10^1$

$= 10^{9+1}$

$= 10^{10}\ EID_{50}$ per mL

The volume of one dose is 30 µL. Therefore:

$$\text{Titre of 'wet' vaccine per dose} = \frac{10^{10}\ EID_{50} \times 30\ \mu L}{1\ mL}$$

$1\ mL = 1000\ \mu L$

$$\text{Titre of 'wet' vaccine per dose} = \frac{10^{10}\ EID_{50}/mL \times 30\ \mu L}{1000\ \mu L}$$

$= 10^{10}\ EID_{50} \times 0.030$

$= 10^{10} \times 10^{-1.5}$

$= 10^{10+(-1.5)}$

$= 10^{8.5}\ EID_{50}$

	Titre after storage					
	Day 0	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
EID_{50} per mL	9	7.8	6.8	6.8	6.5	5.8
EID_{50} per dose	8.5	7.3	6.3	6.3	6.0	5.3

(b) The minimum field dose of vaccine is $10^6\ EID_{50}$ per dose. This vaccine may be used for no more than 8 weeks if it is kept at temperatures below 22°C.

(c) Volume of wet I-2 ND vaccine $= 25\,000$ doses $\times$ 30 µL

$= 25\,000$ doses $\times$ 0.030 mL

$= 750$ mL

The vaccine is prepared by mixing 1 part of allantoic fluid with 1 part of 1% gelatin in PBS.

Volume of allantoic fluid in 750 mL wet I-2 ND vaccine $= 0.5 \times 750$

$= 375$ mL

$$\text{Number of eggs needed} = \frac{375\ mL\ \text{allantoic flui}}{5\ mL\ \text{per egg}}$$

$= 75$ eggs

A minimum of 75 fertile eggs must be inoculated to produce 25 000 doses of vaccine. In practice, always incubate and inoculate more eggs to allow for non-specific embryonic death and inoculation errors.

2. (a) Titre per dose:

	Titre after storage					
	Day 0	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks
EID_{50} per mL	9.8	8.7	8.3	7.5	7.2	6.2
EID_{50} per dose	8.4	7.3	6.9	6.1	5.8	4.8

(b) The minimum field dose of vaccine is 10^6 EID_{50} per dose. This vaccine may be used for no more than 6 weeks if it is kept at temperatures below 22°C.

(c) A minimum of 320 fertile eggs must be inoculated to produce 80 000 doses of vaccine. In practice, always incubate and inoculate more eggs to allow for non-specific embryonic death and inoculation errors.

4.4.2

1. $10^{7.7}$ EID_{50}/0.1 mL, $10^{8.7}$ EID_{50}/1 mL or $10^{7.2}$ EID_{50}/dose. The titre of this vaccine exceeds the minimum production titre.

2. $10^{8.3}$ EID_{50}/0.1 mL or $10^{9.3}$ EID_{50}/1 mL or $10^{7.8}$ EID_{50}/dose. The titre of this vaccine exceeds the minimum production titre.

3. $10^{7.5}$ EID_{50}/0.1 mL or $10^{8.5}$ EID_{50}/1 mL or $10^{7.0}$ EID_{50}/dose. The titre of this vaccine exceeds the minimum production titre.

4. $10^{6.4}$ EID_{50}/0.1 mL or $10^{7.4}$ EID_{50}/1 mL or $10^{5.9}$ EID_{50}/dose. The titre of this vaccine is too low to meet the minimum production titre and the vaccine should be rejected.

5. $10^{6.5}$ EID_{50}/0.1 mL or $10^{7.5}$ EID_{50}/1 mL or $10^{6.0}$ EID_{50}/dose. The titre of this vaccine is too low to meet the minimum production titre and the vaccine should be rejected.

The Standard Error (SE) of infectivity titres calculated in this manner can be high as they are derived from tenfold dilutions and using only five samples per dilution. However, for all practical purposes there is little to be gained by using smaller dilution factors or by increasing the number of samples to reduce the SE.

4.8.3

1. The positive and negative controls on the last plate (rows E and F, G and H) show the expected HI titres (2^5 and 0). Therefore, the dilution of the ND virus antigen was correct and the HI test is working well.

There is no agglutination in any well of column 12 of any plate. Therefore, all sera that were tested do not have natural agglutinin. Natural agglutinins can interfere with the HI reading if the HI titre of the serum is low. If any of the wells in column 12 had shown agglutination, the serum needs to be adsorbed to remove the natural agglutinin (see Section 4.8.6), and then retested.

2. and 3.

Serum No.	
1	HI titre is 2 (2^1). The titre is not considered to be protective.
2	HI titre is 64 (2^6). The titre is considered to be protective.
3	HI titres are 32 (2^5) and 2 (2^1). Duplicate samples are inconsistent. The test must be repeated.
4	HI titre is 4 (2^2). The titre is not considered to be protective.
5	HI titre is 8 (2^3). The titre is considered to be protective.
6	HI titre is 0 (2^0). The titre is not considered to be protective.
7	HI titre is 128 (2^7). The titre is considered to be protective.
8	HI titre is $>2^{11}$. The titre is exceptionally high.
9	HI titres are 64 (2^6) and 32 (2^5). Duplicate samples are inconsistent. However, both results are $>2^3$ so the titre is considered to be protective.
10	HI titres are 2 (2^1) and 4 (2^2). The titre is not considered to be protective.
11	HI titre is 1024 (2^{10}). The titre is considered to be protective.
12	HI titre is 16 (2^4). The titre is considered to be protective.
13	HI titres are 0 and 2 (2^1). Duplicate samples are inconsistent. However, both results are $<2^3$ so the titre is not considered to be protective.
14	HI titres are 8 (2^3) and 4 (2^2). Duplicate samples are inconsistent. However, both results are $<2^3$, so the titre is not considered to be protective.

4. If the chicken comes from a vaccinated flock, it may not have received enough vaccine, the vaccine may not have been effective, the blood sample may have been taken too early after vaccination or the chicken may have been incapable of mounting an adequate immune response (perhaps due to parasitism or malnutrition). Some of the reasons for vaccine failure are discussed in Appendix 8 of *Controlling Newcastle disease in village chickens: a field manual* (Alders and Spradbrow 2001).

5. The inconsistency could be caused by differences in volume between the two samples — too much serum taken in the first sampling, or too little serum in the second sampling. The reason could be a faulty pipette, blocked pipette tips or operator error. Therefore, if there is still serum remaining or if more serum can be obtained, repeat the test. If the test cannot be repeated and a judgment has to be made, then the chicken probably can be considered as having protective antibody.

6. The reading for sample 8 must be accepted as correct because all controls are correct. This high titre suggests that the chicken had been exposed to a virulent field strain of ND virus or had been vaccinated with inactivated or live mesogenic ND vaccine. The serum sample requires further dilution to determine its titre.
7. Serum No. 9: The results of the duplicate samples are inconsistent. This could be caused by pipette error. Repeat the test. If this is not possible, a judgment can be made. The titre of the pair is 2^5 and 2^6. Thus, it is most probable that the chicken has protective antibody.

   Serum No. 10: Repeat the test if possible or if insufficient serum remains, make a judgment. Since both results are $<2^3$, the titre is not considered protective.

   Serum No. 13: Repeat the test if possible or if insufficient serum remains, make a judgment. Since both results are $<2^3$, the titre is not considered protective.

4.9.3

1. From Section 4.9.2
   Flock size 50 birds — 30 birds should be sampled
   Flock size 100 birds — 40 birds should be sampled
   Flock size 200 birds — 50 birds should be sampled.
2. If possible, collect 20 serum samples from vaccinated birds and an additional 20 serum samples from non-vaccinated birds in the same village. Perform HI tests on the sera and then compare the results using an unpaired 't' test to determine whether the difference in titres is significant. If only vaccinated birds are available for sampling, follow the protocol given in Section 4.9.2.
3. Single-point serological surveys are difficult to interpret, especially where ND is concerned. If possible, it is always useful to take paired serum samples to see if the titres to ND are changing, indicating an active disease process. In addition, when virulent strains of ND cause outbreaks in a village, most susceptible birds will die. Birds that are found in a village after such an outbreak will either have:
   - extremely high antibody titres indicating that they had some immunity to ND and were able to mount an effective immune response, or
   - low antibody titres indicating that they did not come into contact with the ND virus.

N.B. It is usually possible to differentiate between vaccinated birds and those birds that have been in contact with virulent field strains of ND. Vaccinated birds will have titres between $\log_2$ 3–8, with naturally infected birds having titres as high as $\log_2$ 10–12 or more.

5.10

Dilution	HA results	No. of infected eggs	No. of uninfected eggs	Accumulated total infected eggs	Accumulated total uninfected eggs	Ratio and % accumulated infected eggs	
10^{-1}	+ + + + +	5	0				
10^{-2}	+ + + + +	5	0				
10^{-3}	+ + + + +	5	0				
10^{-4}	+ + + + +	5	0				
10^{-5}	+ + + + +	5	0				
10^{-6}	+ + + + +	5	0				
10^{-7}	+ + + + +	5	0	12	0	12/12	100%
10^{-8}	+ + + + –	4	1	7	1	7/8	87%
10^{-9}	+ + + – –	3	2	3	3	3/6	50%
10^{-10}	– – – – –	0	5	0	8	0/8	0%

Titre of vaccine = 10^9 EID_{50}/mL.

The recommended minimum dose is 10^6 EID_{50} per bird. Therefore, for the vaccine to be effective it must contain at least 10^6 EID_{50} per drop — that is, 10^6 EID_{50} per 40 µL.

1 mL divided by 40 µL = 25

Therefore, 25 drops make 1 mL.

$$\begin{aligned} 25 \text{ drops/mL} \times 10^6 \text{ EID}_{50}\text{/drop} &= 25 \times 10^6 \text{ EID}_{50}\text{/mL} \\ &= 2.5 \times 10^7 \text{ EID}_{50}\text{/mL} \\ &= 10^{0.4} \times 10^7 \text{ EID}_{50}\text{/mL} \\ &= 10^7 \text{ EID}_{50}\text{/mL} \end{aligned}$$

Since the titre of the vaccine is greater than the recommended minimum field titre, the field staff can use this vaccine.

Appendix 12
Newcastle disease in Australia

Velogenic ND was first reported in Australia in 1930 and 1932. The outbreaks were confined to the suburbs of Melbourne in Victoria, and on both occasions, the disease was successfully eradicated by slaughter (Johnstone 1933; Albiston and Gorrie 1942).

In 1966, an avirulent strain of ND virus was identified in Australia. It was isolated from the proventriculus of a broiler chicken that was diagnosed with a nutritional deficiency and a concurrent infection with *Staphylococcus* (Simmons 1967). Subsequent studies showed that ND virus was widespread throughout Australia, and lacked pathogenicity for chickens (Westbury 1981). The prototype strain was designated V4. Avirulent strains of ND virus are now endemic in poultry flocks in Australia and are also present in a number of species of wild birds (Westbury 1979; Spradbrow, MacKenzie and Grimes 1995). During the 1990s, strains of ND virus were isolated from broiler chickens showing clinical signs of a mild disease, termed late respiratory syndrome. SPF chickens infected with these isolates showed no clinical signs or gross lesions, but histological evidence of mild tracheitis was found (Hooper et al. 1999).

A number of outbreaks of Newcastle disease occurred in Australia from September 1998 to October 2002. In most cases the number of farms involved was small and the high rates of morbidity and mortality and distinctive clinical signs usually seen with exotic ND outbreaks were often not seen. Genetic sequencing of the viruses responsible for the outbreaks showed that they had originated from an avirulent ND virus of Australian origin, genetically distinct from other Australian ND viruses (V4 and V4-like viruses), and from ND viruses that occur overseas. Outbreaks were successfully eradicated using processes prescribed in Australia's AUSVETPLAN disease strategy for the control and eradication of ND. Vaccination using live V4 and inactivated vaccine was introduced to reduce the risk of circulating precursor ND viruses mutating into virulent forms (Animal Health Australia 2010).

In January 2001, a national survey was conducted to collect information on the type and distribution of ND viruses in Australian poultry flocks. The survey detected no virulent ND virus, and no ND viruses similar to the viruses associated with the New South Wales outbreaks. All ND viruses detected were V4 virus or V4-like viruses genetically remote from virulent ND virus (Kite, Boyle, Pritchard, Garner and East 2007).

The current *National Newcastle Disease Management Plan 2008–2012* lays out a program based on the level of risk for an outbreak of virulent ND within Australia. In low risk states, reduced vaccination is permitted but on-going surveillance must be conducted to assess the epidemiology of ND virus.

The outbreaks of ND in Australia were not the first reported instance of possible dynamic shifts in ND virus populations. In 1990, ND was diagnosed in two flocks of laying chickens in Ireland (Alexander et al. 1992). Virulent viruses isolated from these outbreaks were very closely related antigenically and genetically to strains of low virulence. Such low-virulence strains were normally isolated from feral waterfowl but were known to have infected chickens in Ireland in 1987 (McNulty et al. 1988). This virus group was shown to be antigenically and genetically distant from all other ND viruses and it was suggested that the virulent viruses had arisen from those of low virulence.

How such shifts in virus populations occur has not been proven. However, it is known that the genetic material (genome) of all organisms, including viruses, is subject to alteration by mutation. Ribonucleic acid genomes seem more susceptible to such alterations (as the genome itself is replicated) than are deoxyribonucleic acid genomes of other viruses or of other organisms (Spradbrow 1992). During replication, progeny viruses with variant genomes may be produced. These will remain unnoticed in the virus population until selection pressures such as changes in the environment (for instance, intensification of poultry industries) or host characteristics ('improved' breeds, altered disease or immune status) favour a shift in the predominant virus type in the population (Westbury 2001).

Appendix 13
Sources of further information

Dr Joanne Meers
Associate Professor of Veterinary Virology
School of Veterinary Science
The University of Queensland
Gatton QLD 4343
Australia
Tel: +61 7 5460 1839
Fax: +61 7 5460 1922
Email: j.meers@uq.edu.au

International Rural Poultry Centre, KYEEMA Foundation
GPO Box 3023
Brisbane QLD 4001
Australia
Tel: +61 7 3025 8500
Fax: +61 7 3025 8555
Email: ruralpoultry@kyeemafoundation.org
Website: http://www.kyeemafoundation.org

Animal Health Coordinator, The Australian Centre for International Agricultural Research
GPO Box 1571
Canberra ACT 2601
Australia
Tel: +61 2 6217 0500
Fax: +61 2 6217 0501
E-mail: aciar@aciar.gov.au
Website: www.aciar.gov.au

More information is available from the following websites:

- **International Rural Poultry Centre:** http://www.kyeemafoundation.org/content/irpc.php
- **Australian Centre for International Agricultural Research:** http://www.aciar.gov.au
- **World Organisation for Animal Health:** http://www.oie.int
- **International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products:** http://www.vichsec.org

ACIAR

Research that works for developing countries and Australia

aciar.gov.au