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Temperature sensitivity of vaccines

Immunization, Vaccines and Biologicals

World Health Organization

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Contents

Acknowledgement

The first version of this document was developed by Artur Galazka in 1989, as a WHO publication (WHO/EPI/GEN/89.08) called Stability of vaccines (*49*). The current document is a revision of the classic document, also by Dr Galazka, with the assistance of Julie Milstien and Michel Zaffran, entitled Thermostability of Vaccines (WHO/GPV/98.07), which was based on that earlier work. It has been updated by Julie Milstien, Ümit Kartoglu and Michel Zaffran to include both new products and new strategic practices. The authors are indebted to Dr Galazka, who provided the energy and the inspiration for this effort.

The authors would like to express their thanks to the vaccine manufacturers who shared their unpublished thermostability data with us and commented on the text, and to staff of the Program for Appropriate Technology in Health (PATH) for their constructive comments.

Abbreviations and acronyms

°C	degree Celsius
ADT	accelerated degradation test
Al	aluminum
$AlPO_4$	aluminum phosphate
BCG	bacille Calmette-Guérin (vaccine)
$CCID_{50}$	cell culture infectious dose to kill 50% of cells
CO2	carbon dioxide
CP	culturable particle
D2O	deuterium oxide (heavy water)
DNA	deoxyribonucleic acid
DT	diphtheria-tetanus vaccine
DTP	diphtheria and tetanus toxoid and pertussis vaccine
EEFO	earliest expiry first out
ELISA	enzyme-linked immunosorbent assay
EPI	Expanded Programme on Immunization (WHO)
EVSM	WHO-UNICEF Effective Vaccine Store Management initiative
eIPV	enhanced IPV
GTN/VM	Global Training Network on Vaccine Management
HepB	hepatitis B vaccine
HBsAg	hepatitis B surface antigen
HDCV	human diploid cell rabies vaccine
Hib	*Haemophilus influenzae* type b conjugate vaccine
IPV	inactivated polio vaccine
IU/ml	international units per milliliter
JE	Japanese encephalitis type B vaccine

Lf	*Limes flocculatum* (a measure of flocculating units for toxoid vaccines)
Mfr	manufacturer
MgCl2	magnesium chloride
MMR	measles, mumps, rubella vaccine
MR	measles, rubella vaccine
MVDP	multi-dose vial policy
OPV	oral polio vaccine
PATH	Program for Appropriate Technology in Health
PCEV	purified chicken embryo cell rabies vaccine
PFU	plaque-forming unit
pH	hydrogen potential (measure of acid content of a solution)
Td	tetanus and diphtheria (reduced component) toxoid vaccine
TT	tetanus toxoid
UNICEF	United Nations Children's Fund
VVM	vaccine vial monitor
WHO	World Health Organization
YF	yellow fever vaccine

Introduction

More than two million deaths were averted by immunization, as well as an additional 600,000 hepatitis-B-related deaths that would otherwise have occurred in adulthood (from liver cirrhosis and cancer) (*152*), attributable to work of national immunization programmes. However, despite this, more deaths could be prevented and illnesses avoided, if vaccines which are sensitive both to excessive heat and excessive cold, were transported and stored correctly (*34, 75*).

Emphasis is being increased on vaccine management to protect vaccines from both heat and cold. New tools and new training initiatives are being developed for this purpose (*177*). A knowledge of a vaccine's stability, especially of the rate of loss of characteristics that make it safe and effective, with time of exposure to temperatures outside of the 2-8°C range, can help immunization managers better run these programmes.

This version of the document incorporates detailed information on vaccine management related issues and especially concerns on exposure of freeze sensitive vaccines to freezing temperatures. Vaccine vial monitor information has also been expanded to explain how different categories apply to different types of vaccines. The document also discusses the future of the cold chain in reducing the dependency by exploiting the stability of each vaccine to the greatest possible extend. Detailed information is also included on the use of shake test along with provision of a learning guide as an annex.

Part I describes the issues related to vaccine stabilization, with emphasis on the projected evolution of the cold chain to protect vaccines both from heat and cold exposure. Part II details the stability of vaccines that are currently commonly used in national immunization programmes. It includes several vaccines not included in the previous document, products with heightened stability characteristics, new combination vaccines which may behave differently in terms of temperature sensitivity compared to the individual components, and products from an increased number of manufacturers, including many from developing countries. Part III focuses on other vaccines which are being used in some immunization programmes, and may be more used in the future. Part IV provides a summary taking into consideration the future evolution of the cold chain and of future vaccines, and attempting to predict applicability of the information provided here to the immunization worker in the field.

Part I:
Issues related to vaccine stability

1. Ensuring the optimal potency of vaccine: Effective vaccine management practices

To ensure the optimal potency of vaccines, careful attention is needed in handling practices at the country level. These include storage and transport of vaccines from the primary vaccine store down to the end-user at the health facility, and further down at the outreach sites.

The recommended conditions for storing vaccines used in immunization programmes are shown in Figure 1. This diagram also indicates the maximum times and temperatures in each case. At the higher levels of the cold chain, i.e., at national (primary), and regional or province level, OPV must be kept frozen between -15°C and -25°C. Freeze-dried vaccines (i.e., BCG, measles, MMR and yellow fever) may also be kept frozen at -15°C to -25°C if cold chain space permits, but this is neither essential nor recommended. At other levels of the cold chain (intermediate vaccine stores and health facilities), these vaccines should be stored between +2°C and +8°C. All other vaccines should be stored at between +2°C and +8°C at all levels of the cold chain *(149, 150)*. Liquid formulations of vaccines containing diphtheria, pertussis, tetanus, hepatitis B, *Haemophilus influenzae* type b, IPV and their combinations should not be frozen.

Figure 1: WHO recommended vaccine storage conditions[1]

<table>
<tr><th rowspan="2"></th><th>Primary vaccine store</th><th colspan="2">Intermediate vaccine store</th><th rowspan="2">Health centre</th><th rowspan="2">Health post</th></tr>
<tr><th></th><th>Region</th><th>District</th></tr>
<tr><td>OPV</td><td colspan="2">-15°C to -25°C</td><td colspan="3" rowspan="14">All vaccines are recommended to be stored at +2°C to +8°C</td></tr>
<tr><td>BCG</td><td colspan="2" rowspan="7">WHO no longer recommends that freeze-dried vaccines be stored at -20°C. Storing them at -20°C is not harmful but is unnecessary. Instead, these vaccines should be kept in refrigeration and transported at +2°C to +8°C.</td></tr>
<tr><td>Measles</td></tr>
<tr><td>MMR</td></tr>
<tr><td>MR</td></tr>
<tr><td>YF</td></tr>
<tr><td>Hib freeze-dried</td></tr>
<tr><td>Meningococcal A&C</td></tr>
<tr><td>HepB</td><td colspan="2" rowspan="7">+2°C to +8°C
These vaccines are freeze sensitive and must never be frozen</td></tr>
<tr><td>IPV</td></tr>
<tr><td>DT</td></tr>
<tr><td>DTP</td></tr>
<tr><td>DTP-HepB</td></tr>
<tr><td>Hib liquid</td></tr>
<tr><td>Td</td></tr>
<tr><td>TT</td></tr>
<tr><td colspan="6">Diluent vials must NEVER be frozen. If the manufacturer supplies a freeze-dried vaccine packed with its diluent, ALWAYS store the product at between +2°C and +8°C. If space permits, diluents supplied separately from vaccine may safely be stored in the cold chain between +2°C and +8°C.</td></tr>
</table>

Vaccine management encompasses activities related to handling of vaccines at the country level from the moment they arrive till the moment they are used. It includes arrival and acceptance procedures, appropriate temperature monitoring, ensuring sufficient storage volume, maintaining standards of building, equipment and vehicles, effective stock management, vaccine delivery systems as well as effective use of policies such as the multi-dose vial policy (MDVP) and the use of vaccine vial monitors (VVM). Standard tools exist from WHO and UNICEF to effectively monitor management performance of vaccine stores and the vaccine management system in a country *(176, 179-183)*.

[1] Applies to WHO prequalified vaccines

Assessments conducted in various parts of the world, using the above-mentioned standard tools have indicated that there were still weak points in vaccine management performance and more attention should be paid to it at all levels. The findings of over 40 assessments conducted between 2002-2005 highlighting the areas for improvement can be summarized as follows:

- Insufficient vaccine arrival procedures are used to document the quality at the time of arrival
- Although staff understand which vaccines should be kept at what temperature, maintaining equipment at the temperature range recommended by WHO is not always observed. In addition, in the case of such violations, proper follow-up actions are not taken. Many countries still lack appropriate temperature monitoring devices for primary and intermediate stores.
- With the introduction of new vaccines and demanding campaigns of measles and tetanus, many countries begin to lack sufficient cold storage capacity.
- Equipment is aged and needs to be replaced.
- Stock management systems need improvement. Insufficient systems result in expiry of vaccines during their storage. The "Earliest expiry first out" (EEFO) principle is not observed at all times.
- Vaccine distribution remains as one of the greatest risk practices for vaccine quality. Freeze-sensitive vaccines are still carried with frozen ice packs and/or improperly conditioned ice packs risking that they will be exposed to freezing temperatures. Lack of compliance with proper conditioning of ice packs is the universal underlying cause.
- Standard operating procedures for handling of vaccines at the country level have not become part of the vaccine management culture.
- Financial and human resources are insufficient or disproportional to support the work needed.
- The MDVP and VVMs are not used to their utmost potential.

The above picture should be taken as the work load ahead of all partners involved in immunization. The WHO-UNICEF Effective Vaccine Store Management (EVSM) initiative and the Vaccine Management project in coordination with the Global Training Network on Vaccine Management (GTN/VM) now offer strong follow up programmes to countries to improve their vaccine management performance *(153, 176, 179)*.

Since the EVSM's initiation the Sultanate of Oman (for 2002 and 2003) and the Republic of Moldova (for 2003) have been recognized for achieving over 80% in each of the 10 global criteria.

2. Vaccine vial monitors

Heat impact on vaccines is cumulative. The VVM, which was introduced in 1996 for Oral Polio Vaccine (OPV), became available for all other vaccines in 1999 *(156)*. Today, with few exceptions, all manufacturers attach VVM to their products. At any time in the process of distribution and at the time a vaccine is administered, the VVM indicates whether the vaccine has been exposed to a combination of excessive temperature over time and whether it is likely to have been damaged. It clearly indicates to health workers whether a vaccine can be used. VVMs are designed to meet the vaccine's heat stability curve, allowing a margin of safety (*170*). Correlation between the vaccine vial monitor and vaccine potency was tested with OPV and good correlation was found *(172).*

The basis of the setting of the VVMs in use is the accelerated degradation test (ADT), which is used rather than by estimating loss of potency during long periods of storage at different temperatures. The ADT is described in Box 1.

VVM reaction rates are specific to four different models, relating to four groups of vaccines according to their heat stability at two specific temperature points (See Table 1).

Box 1. The Accelerated Degradation Test

In this test samples are subjected to a range of elevated temperatures at which significant and readily detectable degradation is induced in a relatively short time. The rate at which it occurs is measured and extrapolation is made to the lower temperatures at which vaccines are stored, in accordance with the Arrhenius equation (*139*). The precision with which the ADT predicts degradation rates differs considerably, depending on the range of temperatures used, the number of samples tested and the design of the test. The use of ADT results may be further complicated by the different methods and techniques used for estimating the potency of vaccines.

The determination of virus titers of live attenuated vaccines against poliomyelitis, measles or rubella is a simple procedure. In contrast, the biological assays of bacterial vaccines and toxoids are difficult tests requiring large numbers of animals. Potency is expressed in arbitrarily established units or in doses providing 50% protection. The results of these tests are often subject to wide biological variation and it is difficult to obtain precise data on vaccine deterioration unless it has been substantial.

Vaccines and toxoids are made up of proteins, nucleic acids, lipids and carbohydrates, which undergo changes on exposure to heat. The degradation rate of a vaccine is determined by the storage temperature: the higher the temperature, the more rapid and extensive is the degradation. There are considerable differences between degradation rates. However, the degradation rate (b) is not the only factor determining the residual potency (Yt) of a vaccine: the time (T) for which a vaccine is stored at a given temperature and the initial potency of the vaccine (Yo) also have an influence. The relationship between the three factors is expressed as follows:

$$Yt = Yo - bT$$

The usefulness of this formula is limited because many of those involved in immunization programmes may not know the initial potency of a vaccine. However, knowledge of the degradation rate characteristics for various temperatures and of the time of exposure of a suspect vaccine to a given temperature may help a health worker to decide what to do with it.

Table 1: VVM reaction rates by category of heat stability

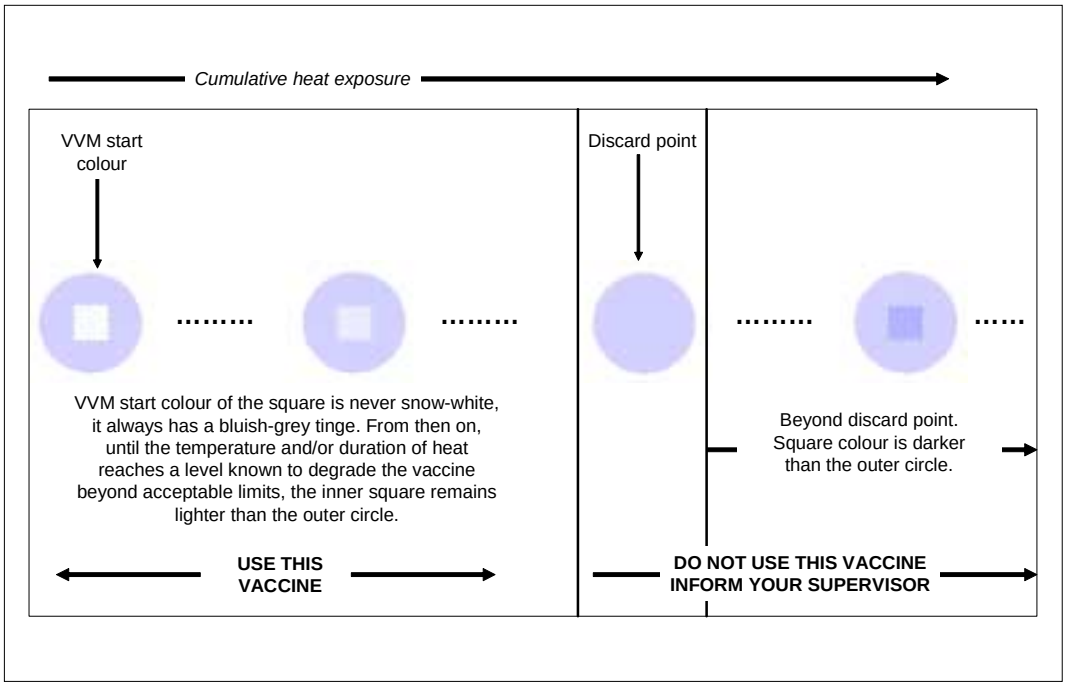
Category: (Vaccines)	No. days to end point at +37°C	No. days to end point at +25°C	Time to end point at +5°C
VVM30 HIGH STABILITY	30	193	> 4 years
VVM14 MEDIUM STABILITY	14	90	> 3 years
VVM7 MODERATE STABILITY	7	45	> 2 years
VVM2 LEAST STABLE	2	NA*	225 days

** VVM (Arrhenius) reaction rates determined at two temperature points*

The reactions of VVMs vary in accordance with the category of vaccine to which they are assigned. VVM2, which is assigned to OPV, the most heat-sensitive vaccine, reaches its end-point in 48 hours (two days) at 37°C, whereas VVM30 on hepatitis B vaccine, one of the most heat-stable vaccines, takes 30 days to reach its end-point at this temperature. However, vaccines made by different manufacturers may have different heat stability characteristics and may therefore be assigned to different categories by WHO. Manufacturer X's BCG might use a VVM30 while manufacturer Y's BCG needs a VVM14.

Reading the VVM is simple; if the inner square is lighter than the outer circle, the vaccine can be used (provided that the expiry date has not passed), and if the inner square is the same colour as or darker than the outer circle the vaccine must not be used. This is illustrated in Figure 2.

Figure 2: How to read a VVM



Vaccines with VVMs can be taken out of the cold chain if health workers and others handling the vaccines have been trained to interpret VVM readings correctly and to discard any vial bearing a VVM that has reached its discard point. Recent studies show that taking vaccines with VVMs out of the cold chain can successfully be implemented without compromising vaccine potency *(97)*. WHO recommends that a policy permitting the use of vaccine outside the cold chain can be implemented either generally for all routine immunization activities or on a limited basis in certain areas or under special circumstances, such as *(151)*:

- national immunization days;
- hard-to-reach geographical areas;
- immunizations provided in the home;
- cool seasons;
- storage and transportation of freeze-sensitive vaccines (DTP, TT, DT, Td, hepatitis B and Hib vaccines) where the risk of freezing is greater than the risk of heat exposure.

3. Prevention of freezing becomes more critical in ensuring vaccine quality

Practices inadvertently exposing vaccines to sub-zero temperatures are widespread in both developed and developing countries and at all levels of health systems *(15, 19, 22, 60, 70, 83, 92 98, 136, 144, 147)*. When a vaccine containing an antigen adsorbed to an aluminium adjuvant (e.g. hepatitis B, tetanus toxoid, ..) is damaged by freezing, the loss of potency can never be restored; the damage is permanent *(34, 35, 40, 75)*.

Freezing affects the adsorbed vaccines through changing their physical form. Freezing does not affect non-potency parameters (such as acid content, pH; flocculating ability (Lf); ratio of free aluminum to aluminum phosphate; free formaldehyde; and thiomersal content). Freezing does affect immunogenicity and texture. Freezing brings changes in the structure and morphology of the adsorbed vaccines, whether monovalent or combined. It has been proposed that ice crystals formed during freezing force aluminum particles to overcome repulsion, thereby producing strong interparticle attraction resulting in aluminum particle coagulation/agglomeration Thus the particles become bigger and these heavy particles sediment faster than particles in never frozen vaccines. The size of the granules seems to increase on repeated freezing and thawing cycles. Adsorbed vaccines kept at the optimal temperature (+2°C to +8°C) show a fine-grain structure under electron and phase-contract microscopy. In contrast, large conglomerates of massed precipitates with a crystalline structure are observed in vaccines affected by freezing. X-ray analysis of vaccines affected by freezing show broad and smeared diffraction lines *(154)*.

Figure 3: Fine-grain structure of aluminum gel stored at the optimal temperature (154)

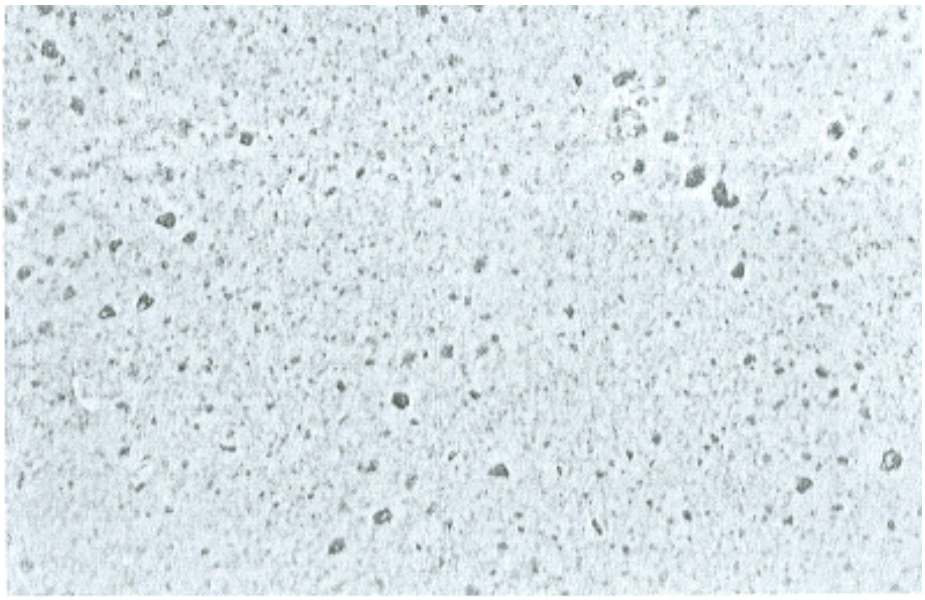
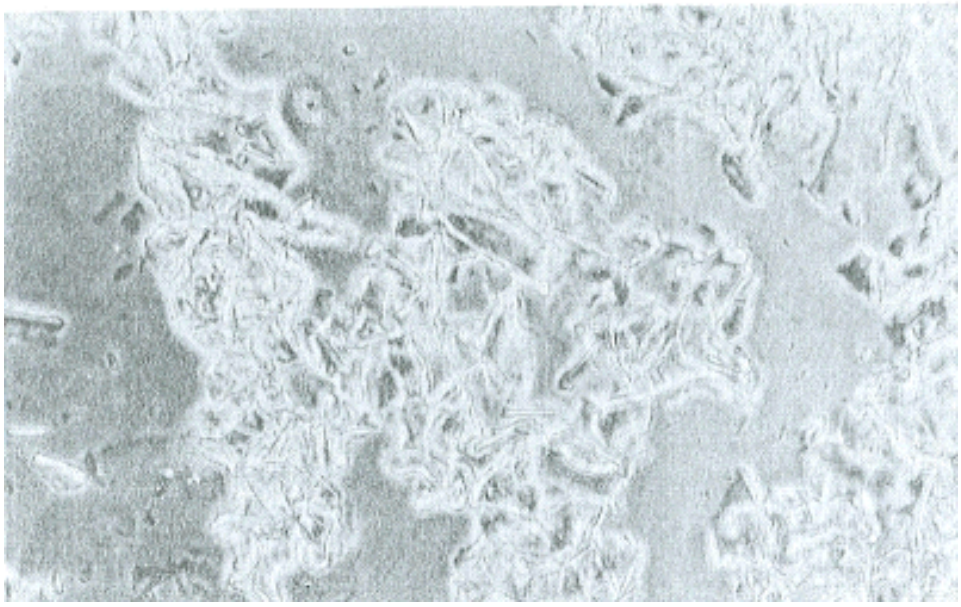


Figure 4: DTP vaccine affected by freezing (at -18°C) showing large conglomerates of massed precipitates with crystalline structure (phase-contrast microscopy) (154)



The physical changes initiated by freezing provide the basis for the shake test. The shake test is designed based on the difference in sedimentation rates of vaccines in frozen and non-frozen vials to understand whether freeze-sensitive vaccines are damaged by freezing *(169)*. A description can be found in Annex 1.

The shake test should NOT be conducted under following circumstances and vials should be discarded immediately, without the need for any confirmatory test:

1) When a solid frozen vaccine vial(s) has been found

2) With a vial for which a homogeneous solution CANNOT be obtained after vigorous shaking as seen in Figure 5. In such cases, the white lump/sediment cannot be separated from the walls of the glass vial. This happens only with DTP vials that are exposed to subzero temperatures without freezing.

Figure 5: Sub-zero temperature effect on DTP vaccine

Photo: Ü. Kartoglu/WHO

Field studies have shown that freeze-sensitive vaccines are exposed to freezing temperatures during their transport in cold boxes from one facility to another *(15, 19, 22 ,60, 70,83, 136, 144, 147)*. The use of deep-frozen ice packs is the main reason for this[2]. WHO has conducted a series of laboratory controlled environment and country studies under real life situations to understand the potential of using cool water packs (cooled at +2°C to +8°C in the main section of the refrigerator) to replace ice during in-country transport *(72)*. Based on the results, investigators defined "cool life" (+2°C to +20°C) as a safety margin such that all vaccines except OPV can safely be transported with cool water packs even in hot climates and up to a repetition of four times *(154)*.

[2] In addition, subzero ambient temperatures may also contribute vaccines being exposed to freezing temperatures during transport if warm packs are not used.

In 2005, WHO established a working group to formulate a new policy statement on prevention of freezing including the introduction of use of cool water packs in vaccine transportation. At the time of preparation of this document, the statement was in draft.

Vaccine discarded due to suspected or known freeze damage as well as heat damage is a preventable cause of vaccine wastage *(154)*. With an increasing number of countries now introducing more costly vaccines that can be damaged by freezing, such as combination vaccines, it is becoming increasingly critical for managers to identify and implement solutions to address such problems.

Although developed as time-temperature indicators, VVMs can contribute significantly to the reduction of vaccine freezing. VVMs make it possible to detect and avoid excessive heat exposure to vaccines when methods are employed to store and transport vaccines without ice and equipment that are known sources of freeze damage *(151)*. VVMs allow health workers to understand that a load of vaccines does not necessarily go bad if the power goes out for a night - they'll be able to see the heat stability of vaccines and begin to accept messages that freezing is a greater danger than mild heat exposure *(171)*.

4. Reducing the dependency: the future of the cold chain

When the management and infrastructure of the Expanded Programme on Immunization (EPI) were being established it was impossible to check whether vaccines retained adequate potency during distribution. Consequently, for the past 20 years, vaccine cold chain systems have been built and maintained on the basis of a single set of rules governing vaccine-handling worldwide, without specific consideration of local environments and types of vaccine. The approach had the merit of simplicity, making the cold chain easy to understand, implement and manage, and presented an uncontroversial concrete objective to be achieved. However, this approach has led to the gradual emergence of a dogmatic view of the cold chain, preventing health workers from taking full advantage of the actual heat stabilities of different vaccines. As a result, the cold chain became too cold.

Immunization programmes have now evolved and diversified: operational strategy reaches out to areas that are difficult to access, large target populations are covered in special campaigns, and a major effort is made to reach every unprotected child. Vaccines have become more stable and there is a clear prospect of increased or even complete heat stability. In these circumstances the dogmatic approach to the cold chain causes resources to be wasted and places unnecessary restrictions on field operations.

The VVM can be seen as a catalyst for much-needed changes in strategies of vaccine distribution via the cold chain. It should eventually allow immunization programmes to exploit the stability of each vaccine to the greatest possible extent, minimize distribution costs, and increase flexibility in the handling of vaccines in the field, thus helping to make operations more effective.

The new definition of "cool life" to allow vaccines being transported with cool water packs will also be one of the revolutionary changes in classic cold chain policies. Countries are more and more convinced by the evidence so far presented and have already introduced changes in this regard. Today, at least three countries in three different continents, Indonesia, Zimbabwe and Moldova have introduced the use of cool water packs for vaccine in-country transport *(9, 50, 97).*

New insulation technologies combined with super-efficient compressors and improved temperature controls promise to radically change the energy requirements for refrigeration *(171)*. The future of the cold chain will also be shaped with such technologies. Improved electronic recording thermometers that are affordable at the health center level will also help in improving the quality of the cold chain. Through such devices, health workers and supervisors will feel more comfortable in knowing all the temperature exposures (and especially low alarms) during long weekends and holidays.

Vaccine distribution without a cold chain would considerably simplify the delivery system and make it easier to integrate with drug distribution in developing countries.

Part II: Analysis of vaccine stability - vaccines commonly used in immunization programmes

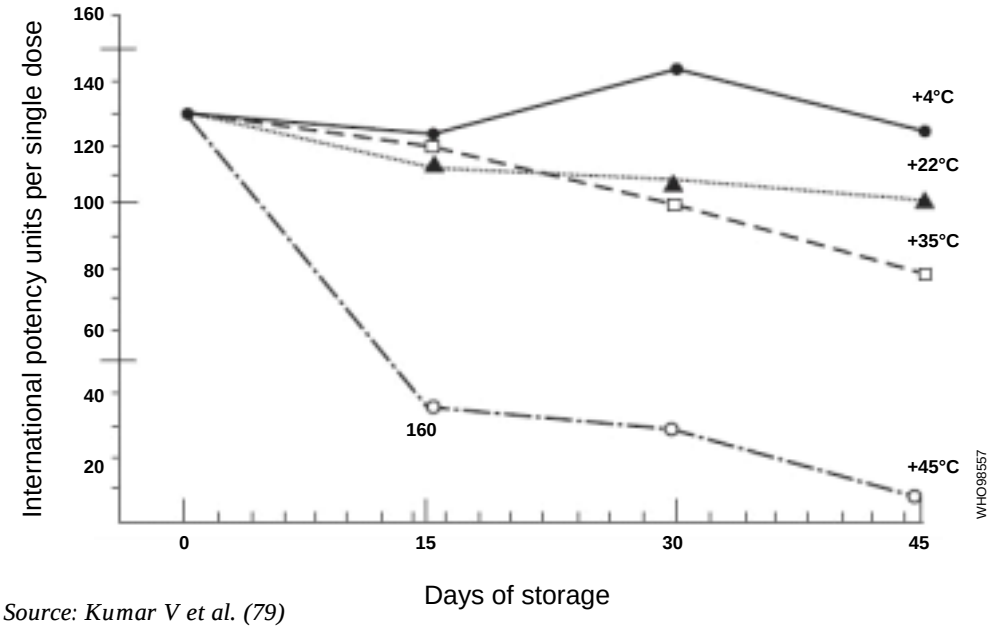
5. Diphtheria and tetanus toxoids

5.1 Exposure to high temperatures

The toxoids of diphtheria and tetanus, partially purified inactivated forms of the corresponding toxins, have stability similar to any simple polypeptide, that is, unaffected by rising temperatures up to the point where secondary structure is lost: generally well above 50°C. In vaccines, these toxoids are present in monovalent form or as components of combined vaccines, adsorbed onto aluminum-based adjuvants. They are stable at elevated temperatures even for long periods of storage. However, they may change their appearance and lose potency when frozen because the adjuvant gel structure is destroyed by freezing. The potency of the tetanus component of adsorbed DTP vaccines does not show significant changes at temperatures in the range 4-8°C for three to seven years (*74*). The shelf-life, at the temperature usually recommended by manufacturers (2-8°C) depends on the nature of the vaccine: the validity period is usually longer for monovalent toxoids or combined diphtheria and tetanus vaccines (usually three years) than for DTP vaccines (18-24 months). In DTP vaccine the limiting factor is the pertussis component; in other combos, it is still generally the least stable component.

The toxoid components of DTP-containing vaccines show an insignificant decrease in potency when stored for 1.5 years at 18°C (*124*), for 6 to 12 months at 24°C (*129*), and for 2 to 6 months at 37°C (*129, 141*). In some DTP vaccines, the tetanus toxoid component showed more accentuated deterioration when stored for 45 days at 22°C and 35°C; the daily losses were about 0.5% and 1%, respectively (*79*). This loss was more pronounced at 45°C (Figure 6).

Figure 6: Potency of tetanus component of DTP vaccine stored for 45 days at various temperatures



Source: Kumar V et al. (79)

At temperatures above 45°C the degradation of toxoid potency is accelerated, and Arrhenius behaviour no longer occurs as the proteins are denatured. After exposures to 53°C lasting four and eight days respectively, monovalent adsorbed tetanus toxoid subjected to the ADT lost 17% and 47% of its initial potency (Table 2) (28). Tetanus and diphtheria toxoids exposed to 60°C are destroyed in three to five hours (*28*, *128*).

Table 2: Potency of adsorbed tetanus toxoid after various periods of storage at different temperatures

Temperature (°C)	Time of exposure (hours)	Remaining potency (percent)
53	96 192	83 53
55	32 72 144 288	97 52 44 35
65	3	20

Source: Cohen H, van Ramshorst JD, Tasman A (28).

Few data are available on the thermal sensitivity of the toxoids in combination vaccines containing HepB and Hib; however, there is no obvious reason to suspect there would be a change in heat stability.

Long exposure to high temperature may result in some changes in the physical characteristics of the aluminum compound which may not be revealed by animal potency tests. The aluminum hydroxide adjuvant showed evidence of 'ageing', in the form of morphological and structural changes, when stored as a single compound or as the adjuvant of diphtheria, DT and DTP vaccines (*2*). A continuous decline in its ability to adsorb Congo red dye was observed during storage at temperatures of 4 to 10°C for 5.5 years. Electron microscope and roentgenographic studies showed that morphological and structural changes progressed more rapidly at 10°C than at 4°C (*187, 188*).

5.2 Exposure to freezing temperatures

Adsorbed vaccines, whether monovalent or combined, alter their physical appearance after freezing has induced changes in the structure and morphology of the adsorbent. Changes in pH and storage at higher temperatures have no influence on the structure of aluminum gel, but freezing causes extensive morphological changes that are visible under the electron microscope (*2*). The development of agglomerates, floccules or other granular matter produces an increase in sedimentation rates (*2, 40, 91, 124*), and the granules do not form a uniform suspension even on vigorous shaking. The size of the granules seems to increase on repeated freezing and thawing (*91*).

The time required to freeze DTP, DT or tetanus toxoid (TT) vaccines depends on the number of doses in the vial (the greater the volume, the longer the time) and on the temperature: 110 to 130 minutes at -10°C, 25 to 45 minutes at -20°C, and 9 to 11 minutes at -70°C. Because of supercooling, the temperature in DTP, DT or TT vaccine vials falls to well below zero (-1.6°C to -2.6°C when the outside temperature is -4.2°C to -4.6°C) before reaching an unstable threshold. At the moment of solidification the temperature in the frozen vaccine rises to the scientific freezing point, which is about -0.5°C (*44*).

The physical changes induced by freezing provide the basis for the shake test, which can be useful in detecting previous freezing in adsorbed vaccines (*40, 169*). This test is easy to perform: the vaccine container is vigorously shaken along with a vaccine vial from the same manufacturer and lot number which has been frozen overnight at -20°C and then thawed, the contents are examined for physical changes, and the extent of sedimentation is continuously observed, compared to the control vial. A sedimentation rate as fast as or faster than that of the control vial suggests that the vaccine has been frozen. However, performing the test needs some experience (see learning guide, Annex 1). Furthermore, not all vaccines show visual changes after freezing (see below).

If it is suspected that adsorbed DTP, DT, or TT have been frozen they should be examined for physical changes. Where these are found the vaccines should be discarded. The amount of antigen in a non-homogeneous vaccine can vary greatly, and the administration of such a vaccine may be associated with a reduced immune response or an increased incidence of local reactions.

Freezing can reduce the potency of tetanus toxoid to an extent that evidently varies slightly with the composition of the vaccine. The tetanus toxoid component in two of five DTP vaccines stored for 12 hours at -30°C showed a decrease in potency of about 30%, while there was no such decrease in vaccines kept at between -5°C and -10°C. However, the potency of the tetanus toxoid component in adsorbed DT vaccine was reduced after freezing at both -5°C and -30°C (*40*). This difference is undoubtedly due to the adjuvant effect of the pertussis component in the DTP vaccines when the potency is tested by animal assay. The relevance of this observation to protective efficacy is not known.

Real efficacy data are difficult to get as each product has its own particular threshold for freeze damage. This is why the shake test is so important. There is a difference between exposure to freezing temperatures and freezing sufficiently to destroy the potency. Failure to distinguish between exposure to freezing temperatures and loss of potency due to freezing has led to lack of trust in the shake test. Several articles in the literature have demonstrated that exposure to freezing temperatures can result in freezing of vaccines, although often these temperatures must be well below 0°C; and this freezing results in loss of potency for adsorbed vaccines (*40*). A study performed by Serum Institute of India Ltd on their own TT, DT, Td, and DTP vaccines (*122*) using three freeze-thaw cycles gave the results presented in Table 3.

Table 3: Results of freeze-thaw cycles on potency of adsorbed vaccines

Freeze-thaw cycle	Potency remaining (percent)		
	Tetanus	Diphtheria	Pertussis
1	85	94	100
2	39	80	77
3	40	44	45

Source: Serum Institute of India, Ltd, 2002 (122)

In this study, all vaccines tested, DTP, DT, Td, and TT, showed shake-test behavior after a single cycle of freezing indicating that freeze damage had been sustained, and all passed the abnormal toxicity test after all three freeze-thaw cycles, while no other non-potency parameters were affected. Thus for the vaccines studied, the shake test is predictive of loss of potency. On the other hand, data from Connaught Laboratories Ltd (now a part of Sanofi Pasteur) indicated that for 80 vials of DTP and DTP-IPV, despite a loss of potency on freezing, the vials remained visually unchanged (*35*), suggesting that vials known to be frozen should not be used either.

Frozen monovalent tetanus toxoid, especially that frozen four times, stimulated a lower mean response and a lower proportion of high titres than the unfrozen product in young military recruits, although the significance of the differences was unclear. All persons immunized with frozen toxoids, however, acquired protective levels of tetanus antitoxin. Freezing did not seem to affect the immunogenicity of unadsorbed toxoid (which remained less immunogenic than the adsorbed product) (Table 4) (*91*).

Table 4: Immune response of military recruits immunized with frozen and unfrozen adsorbed tetanus toxoid

Toxoid	Treatment	10 days after first dose		10 days after second dose		10 days after third dose	
		%>0.01 IU/ml	Mean in IU/ml	%>0.01 IU/ml	Mean in IU/ml	%>0.01 IU/ml	Mean in IU/ml
Adsorbed on $AlPO_4$	Unfrozen	50	0.07	89	4.0	90	13.5
	Frozen 1x	47	0.07	84	3.0	73	9.7
	Frozen 4x	46	0.05	77	2.4	69	9.2
Non-adsorbed	Unfrozen	50	0.04	27	0.6	21	3.2
	Frozen 1x	50	0.05	36	0.7	34	3.3
	Frozen 4x	54	0.06	30	0.7	21	3.2

Source: Menon PS et al. (91)

In addition, anecdotal data from several countries, such as Pakistan (*44*) and Iraq (*10*), has associated lower efficacy in populations where TT and DTP vaccines have been used after a known freezing incident, or when stock samples routinely failed the shake test.

5.3 Summary

Diphtheria and tetanus toxoids are some of the most stable vaccines in common use. They are stable at temperatures of 2 to 8°C for years, at room temperature for months, and at 37°C for weeks. At the temperature of 45°C the degradation of toxoids is accelerated and their potency can decline during a few weeks. At 53°C toxoids lose potency after few days, and at 60°C they lose potency after just a few hours. Freezing can reduce the potency of adsorbed toxoids, however, it does not seem to affect the immunogenicity of unadsorbed products. The freezing point for adsorbed toxoids is between -5°C and -10°C. Adsorbed toxoids should never be frozen.

6. Hepatitis B vaccine

6.1 Exposure to high temperatures

Hepatitis B (HepB) vaccine is a liquid suspension consisting of purified hepatitis B surface antigen (HBsAg) produced using DNA recombinant technology, adsorbed onto aluminum salt. The protein HBsAg has a stability comparable to that of D and T toxoids; as with these, the risk of freeze damage is the major issue.

At temperatures of 2 to 8°C, HepB vaccine appears to be stable for many years. The upper limits of storage life have not been defined.

Some yeast-derived recombinant DNA HepB vaccines are apparently stable at elevated temperatures. Vaccines from several manufacturers were stored at different temperatures (Table 5). The vaccines tested represented routine production lots. The results show that HepB vaccines stored at 2°C to 8°C are quite stable for up to four years. The data revealed considerable differences between vaccines stored at elevated temperatures.

Table 5: Stability of hepatitis B vaccines as indicated by immunogenicity tests

Manufacturer (Mfr)	Temperature °C				Reference
	2-8	20-26	37	45	
Several Mfrs	4 years	1 year	2-6 months	1 week	(*87*), Mfr data
A	-	-	3 months	-	Mfr data, In vitro potency
B	-	6 months	1 month	-	Mfr data
C	-	-	1 month	1 week	(*140*), human data
D	-	30 days	1 week	3 days	Mfr data

There were no differences in immune responses between healthy persons immunized with a recombinant vaccine heated to 37°C for one week and similar persons given a control vaccine stored at 4°C; the antibody distribution and geometric mean antibody titers were similar in the two groups. The total incidence, severity and types of symptoms were similar in persons immunized with the two vaccines, and no severe reactions were reported (*71*).In another study, recombinant vaccine was studied in healthy volunteers. Using vaccine stored at 4°C for purposes of comparison it was found that heating vaccine for one week at 45°C or for one month at 37°C did not alter reactogenicity or the ability of the vaccine to elicit antibody titers considered protective (*140*).

6.2 Exposure to freezing temperatures

The freezing temperature of HepB vaccine is -0.5° C and freezing destroys potency, a result of destruction of the aluminum lattice (*125, 175*). HepB vaccine should be protected from being frozen; vaccine thought to have been frozen should not be used

In Indonesia, a study found freezing temperatures in 75% of baseline shipments of hepatitis B vaccine (*97*). Other studies have suggested a correlation between freezing of HepB vaccines and lower than expected seroconversion. (*37, 75, 89*).

6.3 HepB vaccine used outside the cold chain

Based on data collected by PATH (*102*) 75% and 100% of hepatitis B vaccines were exposed to freezing temperatures during storage and distribution in the two developing countries studied. To investigate how to avoid this, a study was conducted in China to assess whether HepB vaccine labeled with VVMs stored outside the cold chain for up to three months could remain effective for delivery to infants at birth (5, *101*). The vaccine, stored at room temperature, was given to 358 infants at birth by village midwives. As a control the same vaccine, stored in a refrigerator, was administered to 232 infants within 24 to 72 hours after birth by village doctors. The second and third doses were given with other vaccines as part of the mobile outreach services, which were available at intervals of about two months. The rates of seroconversion to anti-HBsAg for vaccine stored without and with refrigeration were 81.6% and 81.9% respectively.

A similar study in Indonesia showed that use of HepB vaccine in a pre-filled injection device labeled with a VVM and kept out of the cold chain in Indonesia gave good results in terms of ease of use (*132*) and equivalent seroresponse to vaccines stored in the cold chain (*100*). There is scope for developing a management instruction that would allow removal of the vaccine from the cold chain in emergencies, or in outreach activities of short duration, provided that a high temperature indicator was attached to each vial.

6.4 Summary

These data suggest that HepB vaccine is in the upper range of heat stability, together with tetanus and diphtheria toxoids, among the vaccines commonly used in immunization programmes. The vaccine is stable for up to four years at temperatures of 2 to 8°C, for months at 20°C to 25°C, for weeks at 37°C and for days at 45°C. As with other vaccines adsorbed on aluminum salts, freezing of HepB vaccine may cause a significant reduction of potency. The freezing point of HepB vaccine is about -0.5°C. The vaccine should always be protected from being frozen, especially at the end of the cold chain when it is transported in cold boxes and may come into close contact with cold packs. Freeze damage is the greatest threat to its integrity, and strategies to mitigate the risk of freezing should be employed.

7. Pertussis vaccines

Stability studies on pertussis vaccine are hampered by the lack of a simple, inexpensive and reproducible potency test. The potency test recommended by WHO (*159*) is technically difficult and requires highly qualified staff and a large number of mice of a specific strain. The results are subject to wide biological variation. It is difficult to obtain precise data on the deterioration of the potency of vaccine exposed to elevated temperatures unless it shows marked changes.

Nevertheless, several studies have provided valuable information on various factors that influence the stability of pertussis vaccine. The most frequently studied factors are:

- the temperature (*79, 141*);
- the form of the vaccine: monovalent vaccine versus the pertussis component of DTP vaccine (*4, 33*);
- the method of inactivation (*73*);
- the nature of the adjuvant or preservative (*141*).

Although most developing country immunization programmes use whole cell pertussis vaccines, and the majority of data presented here is for vaccines containing this component, data on the stability of the acellular pertussis compoenent is presented in section 7.1.

7.1 Influence of temperature on vaccine potency and toxicity

The potency of the pertussis component of DTP vaccine depends on the storage temperature; potency may be reduced either by high temperatures or by freezing. The impact of various ambient temperatures on the potency of the pertussis component of DTP vaccine is shown in Table 6.

When stored in a refrigerator between 4°C and 6°C, the pertussis component of DTP vaccines appears to have satisfactory potency over a period of two years (*74*). However, even under optimal conditions, a continuous decrease in potency occurs during long periods of storage. DTP vaccines with an estimated average initial potency of 8.5 IU per single human dose have a potency below 4 IU per dose after 46 months (*74*).[3]

[3] *For acellular pertussis vaccines, a stability profile similar to that of other protein vaccines is to be expected, i.e., relatively good thermostability, poor resistance to freezing and a shelf-life of two to three years at 2 to 8°C (76). A study by Boros et al (20) looked at freezing at -3°C of both acellular and whole-cell pertussis antigens in a triple (DTP) formulation and found that 24 hours exposure to this low temperature gave a reduction of post-immunization IgG responses to the relevant antigens in a murine model.*

Table 6: Stability of the pertussis component of DTP vaccines at various temperatures

Storage temperature (°C)	Reference	Estimated potency loss per day (percentage)	Time of storage and time used for calculation of degradation rates
4-8	33	0.06	6 years
	79	0	45 days
	4	0	12-18 months
	58	0.01	90 days, 15-90 days
	78	0.05-0.06	3 years
22-25	79	0.31	45 days, 0-45 days
	41	0.41	140 days, 40-140 days
	4	0	30 days followed by 18 months at 4°C
	57	0.26	90 days, 15-90 days
30	41	1.80 0.80	90 days, rapid decrease 0-15 days, slow decrease 30-90 days
35-37	140	3-6*	56 days, 0-7 days
	117	1.2	90 days, 0-15 days
	41	5.2	60 days, 0-20 days
	57	2.4	90 days, 0-15 days
	78	5.5	56 days, 0-7 days
46	77, 78	6.7	56 days, 0-7 days
	41	10/8	20 days, 0-4 days

* Two DTP-polio vaccines with different preservatives

Freezing may thus impair the potency of pertussis vaccines. When DTP vaccines are submitted to freezing at -20°C for 15 days the potency of their pertussis component loses more than 50% of its initial value. The potency of the pertussis component is more impaired by freezing than by storage at elevated temperatures (*4*).

When adsorbed DTP vaccines from five manufacturers were kept for 12 hours at between -5°C and -10°C and between -20°C and -30°C, three of them underwent significant losses in the potency of the pertussis component in both temperature ranges (*40*). Table 3 shows similar data from one manufacturer.

There is no evidence that the toxicity of pertussis vaccine increases with storage, as measured by the mouse weight gain and histamine-sensitizing tests (*4, 23,58*). In fact, vaccine samples kept at 25°C and 35°C for between four weeks and three months showed reduced toxicity (*23,581*).

7.2 Monovalent pertussis vaccines versus the pertussis component of combined vaccines

In one study, monovalent pertussis vaccines were evidently unstable at 4°C: during storage for 18 months some samples lost 58% to 87% of their initial potency (*66*). During the first year of storage, *B. pertussis* bulk suspensions seem to deteriorate more rapidly at 4°C than the pertussis component of DTP vaccines adsorbed on aluminum phosphate, possibly because they lack the protective effect of the toxoid proteins and the aluminum ions in the triple vaccine.

7.3 *Methods of inactivating B. pertussis*

Early studies on the stability of pertussis vaccines prepared from cultures grown and killed by various methods suggest that, of the inactivating agents merthiolate, phenol, formalin and heat, none stands out as definitely superior. During prolonged storage, however, vaccines killed with phenol or formalin become dark in colour and difficult to resuspend, while those killed by merthiolate or heat show little change in appearance.

Early observations of Kendrick (*73*) were confirmed by Gupta et al., who studied the stability of DTP pertussis components prepared with different methods of inactivation (heat, formaldehyde, glutaraldehyde, thiomersal or acetone treatment) (*57*). Stability tests performed after the storage of vaccines at 4°C to 8°C, 25°C and 35°C for 90 days showed no differences in stability attributable to the inactivating agents used.

The work of Gupta et al. (*57*), demonstrates the problems encountered in studies on pertussis vaccines: low reproducibility in vaccine potency estimates and differences in degradation rates of vaccines prepared in the same way. The initial potency of vaccines prepared by different inactivation methods differs considerably, with thiomersal-inactivated vaccines having the highest potency and acetone-treated vaccines being of substandard potency.

7.4 *Summary*

In its usual presentation, DTP with thiomersal and aluminum adjuvant is susceptible to freezing but relatively stable at 2-8°C for two years or more. It is resistant to storage for several months at 22 to 25°C, for several weeks at 37°C, and for less than one week at 45°C. As with most protein-containing vaccines, temperatures higher than 56°C are immediately deleterious.

8. Polysaccharide and conjugated polysaccharide vaccines

8.1 Meningococcal vaccine

Purified meningococcal polysaccharides, and especially group A polysaccharides, are unstable at ambient temperatures because of depolymerization. Polysaccharide antigens readily depolymerize and their relative molecular mass diminishes when they are exposed to ambient temperatures. The degree of polymerization is therefore a useful indicator for assessing both immunogenicity and thermal stability.

Storage at -20°C was recommended for the early polysaccharide vaccines in group A. At that temperature, the rate of depolymerization is negligible. The immunogenicity of meningococcal vaccine is related to the molecular size of the protective antigens, polysaccharides A and C; the antibody response increases with the molecular weight. The discovery that the replacement of sodium chloride by lactose as a menstruum for lyophilization stabilizes polysaccharide vaccines against thermal depolymerization represented a major step in achieving more stable vaccines (*137, 164*). These vaccines are supplied in freeze-dried form. The addition of a stabilizer and achievement of a low moisture content have greatly improved their thermal stability.

Stabilized meningococcal vaccines in the lyophilized state can be stored at refrigerator temperatures for two years (*6, 7*), and in fact some formulations have been seen to be stable for 36-48 months at that temperature (manufacturer's data). Group A polysaccharide vaccine was unaffected by being kept at 20-25°C for 12 days or at 35°C for 3 days (*7*). Group A + C vaccine from one manufacturer, stored at 22°C for 18 months, showed very little depolymerization; at 45°C the group A component reached a critical level of depolymerization after four weeks, while the group C component was stable for 8-10 weeks (*6*). An ACWY polysaccharide vaccine was stable in the lyophilized form for up to six weeks at 60ºC; after reconstitution, its shelf life was only a few days at higher than refrigerated temperature (manufacturer's data).

A vaccine reconstituted with diluent containing 0.25% phenol was reported to be stable when stored at -20°C for two months, at 4°C for four weeks, at 25°C for two weeks, or at 37°C for four days (*6*). Despite its relative stability, reconstituted vaccine should be kept at refrigerator temperatures and should be discarded if not used during the day on which it is reconstituted (*164*).

To date, several meningococcal type C conjugate vaccines are available, and very recently a tetravalent conjugate vaccine ($ACYW_{135}$) has been licensed. The Expert Committee on Biological Standardization has defined the types of testing which must be done (*158*). The polysaccharide component of conjugate vaccines may be subject to gradual hydrolysis at a rate that may vary depending upon the type of conjugate, the type of formulation or adjuvant, the type of excipients and the conditions of storage. Ho and colleagues compared two meningococcal conjugate vaccines from two manufacturers, subjected to temperatures up to 55ºC, as well as repeated cycles of freeze-thawing (*63, 64*). They found a variation in structural stability of the oligosaccharide chains and the protein carrier between the two products.

While neither of the products showed sensitivity of immunogenicity to freeze-thawing cycles, one of the vaccines showed substantial release of free saccharide accompanied by significant reduction in IgG and IgM responses in a model system after exposure to 55ºC. However, both products were stable when stored at the recommended temperatures. One meningitis C conjugate vaccine with a shelf life of two years at 2-8ºC, showed retention of potency as detected by a bactericidal or an enzyme-linked immunosorbent assay (ELISA) method, as well as integrity of the conjugate on exposure to either 2-8ºC or 25ºC for up to 36 months (manufacturer's data).

8.2 Haemophilus influenzae type b vaccine

The stability of *Haemophilus influenzae* type b conjugate (Hib) vaccine, may also depend on the impact of adverse factors on the strength of the linkage between the polysaccharide and the protein carrier. Preliminary results suggest that the lyophilized Hib vaccine (tetanus toxoid conjugate vaccine containing purified polyribosyl-ribitolphosphate capsular polysaccharide, PRP-T) is stable at refrigerator temperatures for 36 months and at 25°C for at least 24 months. Liquid monovalent Hib or liquid DTP-Hib vaccines are stable at refrigerator temperatures for 24 months. In multidose formulation, liquid Hib and DTP-Hib vaccines may be used at a subsequent session, even if they have been opened, according to the WHO Policy Statement on the use of opened vials of vaccine in subsequent immunization sessions (*173*). In one study, Hib in the lyophilized form maintained its release specifications for 36 months at 2-8ºC, for 24 months at 25ºC, and for one month at 37ºC, while the reconstituted form was stable for only five days at 37ºC (manufacturer's data). However, it should be noted that in most cases lyophilized vaccine should not be maintained past six hours after reconstitution (*173*). Stability data have been provided on a totally synthetic liquid Hib vaccine (*142*). It is stable at 2-8ºC for more than 18 months, and at 37ºC for three months (manufacturer's data).

Liquid Hib should never be frozen, especially in combinations with DTP, as freezing may damage the immunogenicity of the product (*148*).

The guidelines published by the Expert Committee on Biological Standardization published in the Technical Report Series outline the characteristics of Hib vaccines (*161*).

8.3 Pneumococcal vaccines

As with the other vaccines in this section, vaccines against pneumococcal disease exist in two forms, unconjugated polysaccharide, and conjugated polysaccharide vaccines. The polysaccharide vaccines contain 23 serotypes dissolved in isotonic saline with either phenol or thiomersal adjuvants *(45*), have a shelf life of 24 months at 2-8ºC, and should not be frozen. Sweeney et al.(*134*) examined the impact of storage on molecular size of the polysaccharide components and found that only a few showed a loss of antigenicity on storage of up to 10% per year, while the remainder lost less than 2% per year.

The only currently licensed pneumococcal conjugate vaccine, a 7-valent vaccine produced by Wyeth, is formulated with aluminum adjuvant (*38*), is a liquid, and should be protected from freezing as for other aluminum adjuvanted vaccines. For long term storage it should be stored at 2-8ºC.

9. BCG vaccine

The standardization of the stability of BCG vaccine and studies on it are complicated by the following factors:

1) Different substrains of BCG at various levels of attenuation are used in vaccine production.
2) There are differences in the manufacturing and testing procedures employed by vaccine producers. The technique and time of cultivating BCG and the nature of the stabilizer are important factors.
3) There are differences in bacterial content and the number of culturable particles (CPs) among products.
4) There is no approved laboratory method for assaying the protective potency of vaccines against tuberculous infection in humans.

The most important element in batch-to-batch quality control is the checking of vaccine viability. This involves determining the number of CPs by means of colony counts on solid medium. The viability test is also of prime importance in assessing the stability of BCG stored in different conditions.

BCG vaccine was the first vaccine for which a WHO requirement for heat stability was established (*168*). An ADT should be conducted on each lot of BCG vaccine. The number of CPs in vaccine incubated at 37°C for 28 days should be not less than 20% of that in the vaccine stored at 4°C (*160*).

9.1 Impact of temperature on the viability of BCG vaccine

BCG vaccine is relatively stable at refrigerator temperatures below 8°C, and most manufacturers give a validity period of more than one year, which is stipulated in the amended 1988 WHO guidelines for this product (*160*). At least one manufacturer gives a validity period of two years (*122*). However, some vaccines can lose 20% to 25% of their original viability during storage for only six months (*130*).

A stabilizer is added to the freeze dried preparation. Vaccine stabilized with monosodium glutamate may be more difficult to reconstitute, while the presence of albumin in the stabilizer may lead to foaming during reconstitution of the vaccine (*93*).

9.2 Stability of vaccines produced from different BCG substrains

All available BCG strains are derived from the one produced by Calmette more than 65 years ago. After the long period of maintenance by culture medium transfers of the original strain there are essential differences between daughter strains. BCG strains are usually classified either as strong, as with the French strain 1172 (Pasteur) and the Danish strain 1331 (Copenhagen), or weak, as with the Japanese strain 172, the Brazilian strain Moreau, and the British strain 1077 (Glaxo). This distinction is based mainly on growth characteristics, residual virulence in animals and reactogenicity in children. The differences may be linked with surface antigenic lipid content and secreted protein (*1*).

There are differences in the heat stability of BCG vaccines prepared by different manufacturers from different substrains (Table 7) (*68, 69*). Japanese vaccine prepared from substrain 172 had a higher thermostability than French (strain 1172), Danish (substrain 1331 Copenhagen) and Polish (substrain Moreau) vaccines. At 37°C the time required for a 50% decrease in viability (CPs/mg) of Japanese vaccine was about 56 days, while for the other vaccines it ranged from 28 to 35 days. At 54°C the Japanese vaccine retained more than 50% of its viability for longer than nine days, while the other vaccines lost more than 50% of their original activities in one to three days. It is not clear if these differences are strain dependent or dependent on the lyophilization and stabilization techniques of the manufacturers, though it is probably the latter.

Table 7: Culturable BCG particles in Japanese, Danish, French and Polish BCG vaccines at various temperatures

Temperature (°C)	Number of days	Number of culturable particles/mg			
		Vaccine			
		Japanese	Danish	French	Polish
4°C	Control	47.86	4.68	7.76	6.17
20°C	28	45.71	3.72	6.31	4.57
	63	44.67	3.02	5.89	3.72
	84	39.81	2.24	4.90	2.75
	112	38.02	2.09	3.47	2.19
37°C	14	46.77	3.80	5.50	4.68
	28	37.15	2.34	4.90	2.69
	35	-	-	-	1.62
	42	-	1.32	1.51	0.55
	56	26.92	-	1.55	-
	84	17.78	2.29	0.41	-
54°C	1	47.86	3.47	2.75	1.66
	3	46.77	1.26	1.07	0.91
	6	39.81	0.98	0.63	0.25
	7	-	0.20	0.22	-
	9	32.36	-	-	-

Source: Janaszek W (68).

A vaccine prepared from the Moscow strain of BCG showed a viability loss of 22-41% during storage at 4°C for 18 months (*122*). At room temperature this strain lost no more than 54-66% viability after storage for six months, and it met the specification for storage at 37°C for one month. Other studies have also shown differences in stability between BCG vaccines (*54, 121* – see also Table 8). However, the differences in thermostability shown in Tables 7 and 8 are likely related to other factors than the substrain, such as lyophilization conditions, stabilizer, and relative humidity in the vial. These differences are reflected in the type of VVM assigned to each manufacturer's BCG product.

Table 8: Viability and heat stability of ten BCG vaccines

Vaccine	Initial numer of CPs (x 10^6/ml)	Viability after storage for 28 days at 37°C		Daily loses of viability (percent) (storage period analysed, days)
		CPs (x 10^6/ml)	Percent of initial number of CPs	
Japanese	27.0	16.6	61	(0-28)
Glaxo	20.1	10.9	54	(0-21)
USSR	7.1	3.6	51	1.7 (0-28)
Connaught	6.9	0.2	3	6.7 (0-14)
Dakar	6.5	1.8	28	3.2 (0-21)
Bilthoven	4.2	1.3	31	4.9 (0-14)
Copenhagen	2.9	1.9	66	2.5 (0-28)
Merieux	2.8	0.3	11	3.3 (0-28)
Pasteur Institute	2.7	1.3	48	1.9 (0-28)
Prague	1.1	0.2	18	5.2 (0-21)

Source: Lugosi L (82)

9.3 Packing BCG vaccines

BCG vaccines require special precautions to ensure sufficient stability. In this connection the most important measures are lyophilization, the use of an effective stabilizer, and proper sealing of vaccine containers.

Increased stability at 4°C and 37°C and higher starting viability values (i.e., better survival rates after freeze-drying) have been observed after changing the composition of the stabilizer and improving the drying method (*47*).

Historically the use of ampoules sealed under vacuum was the most common practice for increasing stability. However, vacuum-sealing is difficult compared to sealing in the presence of inert gas. There were no significant differences between BCG vaccines sealed under vacuum and under nitrogen or carbon dioxide at either 4°C or 37°C (*47*). Most manufacturers now prepare BCG vaccines in vials, and under well-validated conditions, the product is relatively stable. One manufacturer recently provided data showing the stability of such a product filled in amber colored vials and freeze-dried under vacuum, showing no lowering in the viability (*122*). Viable counts for vaccine sealed under nitrogen have been reported to decline more rapidly than those for vaccine sealed under vacuum (*18*). A BCG vaccine sealed under argon seemed to have less stability at 37°C than vaccine sealed under vacuum (*53*).

BCG vaccines in rubber-stoppered vials previously showed a lower stability than those conserved in ampoules (*82, 121*), although this seems not to be the case with currently supplied vaccines that use rubber stoppers.

9.4 *Effect of light on the stability of BCG vaccine*

Freeze-dried BCG vaccines, regardless of their substrain, are sensitive to ultraviolet and fluorescent light. They should be protected from light when used (*80*), and many manufacturers pack them in ampoules made from a substance of low light transmittance, such as amber glass.

9.5 *Stability of reconstituted vaccine*

Reconstituted BCG vaccine is very unstable, must be kept cold, and must be discarded within six hours of reconstitution. The reasons for these precautions are as follows:

1) There is a risk of contamination because BCG vaccine, like other lyophilized live vaccines, does not contain any bacteriostatic agent. For this reason, WHO recommends that reconstituted lyophilized vaccine should be kept cold and discarded at the end of six hours (*173)*

2) There is a loss of potency (*39*).

9.6 *Summary*

Most freeze-dried BCG vaccines are stable at temperatures of 2-8°C for at least two years. At room temperature stability varies; after storage for several months a loss of viability of approximately 30% can be expected. The daily loss of viability in vaccines kept for a few weeks at a temperature of 37°C ranges between 1% and 2%. Reconstituted vaccine is very unstable and at risk of contamination. Once reconstituted, all BCG vaccines should be kept cold and discarded within six hours, regardless of how many doses remain in the vial or ampoule.

10. Measles, mumps and rubella vaccines

10.1 Stability of freeze-dried measles vaccine

In recent years significant progress has been made in improving the heat stability of measles vaccine. The development of an effective stabilizer (*56 ,88, 108*) and the formulation of a WHO requirement for heat stability of freeze-dried measles and measles-containing vaccines (*162, 163*) have made a considerable impact on the quality of measles vaccines on the market. This requirement uses two indices of stability:

1) Freeze-dried vaccine should retain at least 1000 live virus particles in each human dose at the end of incubation at 37°C for seven days; and

2) If, during incubation, the virus titer has been decreased, then it shall have done so by not more than 1 log10 (*162*).

The increased heat-stability under normal working conditions is especially important in the developing world (*61*).

Measles vaccine in its dried form is extremely stable at temperatures below zero (*88*). The dried vaccine stays potent if kept cold and it is not damaged by freezing and refreezing.

The thermal degradation of the second generation measles vaccines is slow (*3, 86*). At 2 to 8°C these improved vaccines maintain minimum potency for more than two years (*17, 108)*.

The enhanced thermostability of the second-generation measles vaccines has been confirmed in the field. Studies in Cameroon showed that two second-generation measles vaccines stored at 37°C for up to 14 days, were able to induce seroconversion in seronegative children (*61*). The product of another manufacturer showed acceptable potency after storage in the lyophilized form at 37ºC for 21 days in a 10-dose vial format (*122*). It is reported that the unreconstituted Merck measles vaccine can retain potency for eight months at room temperatures and four weeks at 37ºC (*131*).

At 54 to 56°C measles vaccine is inactivated rapidly, losing more than 0.65 $\log_{10}$ and 1.3 $\log_{10}$, respectively, during one-day and three-day exposures. The time required for reduction in titer to 1000 CCID50 was about 12 hours (*88*).

10.2. Stability of freeze-dried mumps and rubella vaccines and combinations

The WHO thermostability requirements for mumps and rubella vaccines are similar to those for measles vaccine. At least three containers of monovalent or MMR vaccine are tested by incubation at 37°C for seven days, at the end of which each monovalent vaccine or individual vaccine component is titrated in PFUs or CCID50 after selective neutralization, as necessary, of the other components. The geometric mean infectious virus titer must equal or exceed the required minimum number of infective units per human dose (3 $\log_{10}$), and the geometric mean virus titer must not have decreased by more than 1 $\log_{10}$ infective units during incubation (*163*).

The stabilities of both components of lyophilized measles-mumps vaccine are similar at 4°C, 23°C, 37°C and 45°C. At 37°C, the degradation rate is about 0.01 log10 per day for both components. Half lives are also similar: 4.7 and 5.4 days for the measles and mumps components respectively at 45°C; 12 and 13 days at 37°C, and 71 and 65 days at 23°C (*29*).

In the temperature range 20°C to 56°C, the mumps vaccine component in mumps-rubella and MMR vaccines have degradation rates comparable to those for monovalent mumps vaccine (*88*). The mumps component in MMR vaccines from one manufacturer shows good stability at 37°C for up to 21 days; during a 30-day exposure to 37°C, the mumps component of MMR vaccines lost 0.9 $\log_{10}$, i.e. about 0.03 $\log_{10}$ per day, and half lives were about 10 days (*122*).

Freeze-dried monovalent rubella vaccine and the rubella component of measles-rubella, mumps-rubella and MMR vaccines show low degradation rates. At 37°C the average loss of titer ranges from 0.046 to 0.109 $\log_{10}$ CCID50 per week (*88*). The rubella component of Indian MMR vaccine also shows good stability, the average titer loss per week being about 0.1 log10 $CCID_{50}$, and the half life being more than two weeks (*122*). The rubella component seems to be more stable than the other components of combined virus vaccines.

In the US, where there is no ADT requirement for the MMR vaccines, in contrast to the excellent thermostability of the measles vaccine mentioned above, the Jeryl Lynn vaccine was stable for only one week at 37ºC (*88*); the rubella component lost potency after exposure to 37ºC for three weeks (*88*).

10.3 Stability of reconstituted vaccine

Measles vaccines, even those with enhanced thermostability in dry form, quickly lose their potency when reconstituted and kept at elevated temperatures. Reconstituting vaccine with a warm diluent may be harmful; vaccine reconstituted with the diluent prewarmed to 41°C and then further incubated in the waterbath at that temperature lost half of its original potency after half an hour and 0.5 to 0.7 $\log_{10}$ after one hour (*108*). At 37°C the loss of titer was 0.4 to 0.5 and 0.8 to 1.0 $\log_{10}$ after three and six hours respectively (*42, 108*).

Reconstituted measles, mumps, and rubella vaccines and their combinations must be used in the same immunization session. Measles, mumps and rubella vaccines and their combinations are produced in lyophilized (freeze-dried) form and must be reconstituted before use with diluent provided by the manufacturer. If not, this creates an opportunity for errors to be made in handling of the vaccine (*147*). There is a serious risk when reconstituted vaccine is stored at any temperature for longer than six hours or above 8°C for any period. This is not only because of the lack of potency, but also because of the possibility of contamination of the product, which could cause serious adverse consequences in those being vaccinated. When used, measles vaccine should be protected from elevated temperature and from light (light may inactivate the virus). Reconstituted vaccines must be discarded at the end of each immunization session and should NEVER be kept for use in subsequent sessions (*178).*

10.4 Summary

Measles vaccine and MMR components in lyophilized form are quite stable. They are stable at temperatures below zero and are not damaged by freezing and refreezing. At between 2°C and 8°C dried measles vaccine or MMR maintain minimum potency for more than two years. At room temperature (20°C to 25°C) the minimum required infectivity titer of measles or MMR virus is still retained for at least one month and it can be maintained for at least one week at 37°C.

After reconstitution, measles and MMR vaccine rapidly lose their potency when kept at temperatures above 2-8°C. Reconstituted measles and MMR vaccines should be kept cold during immunization procedures, must be discarded at the end of each immunization session and must never be kept for use in subsequent sessions.

11. Yellow fever vaccine

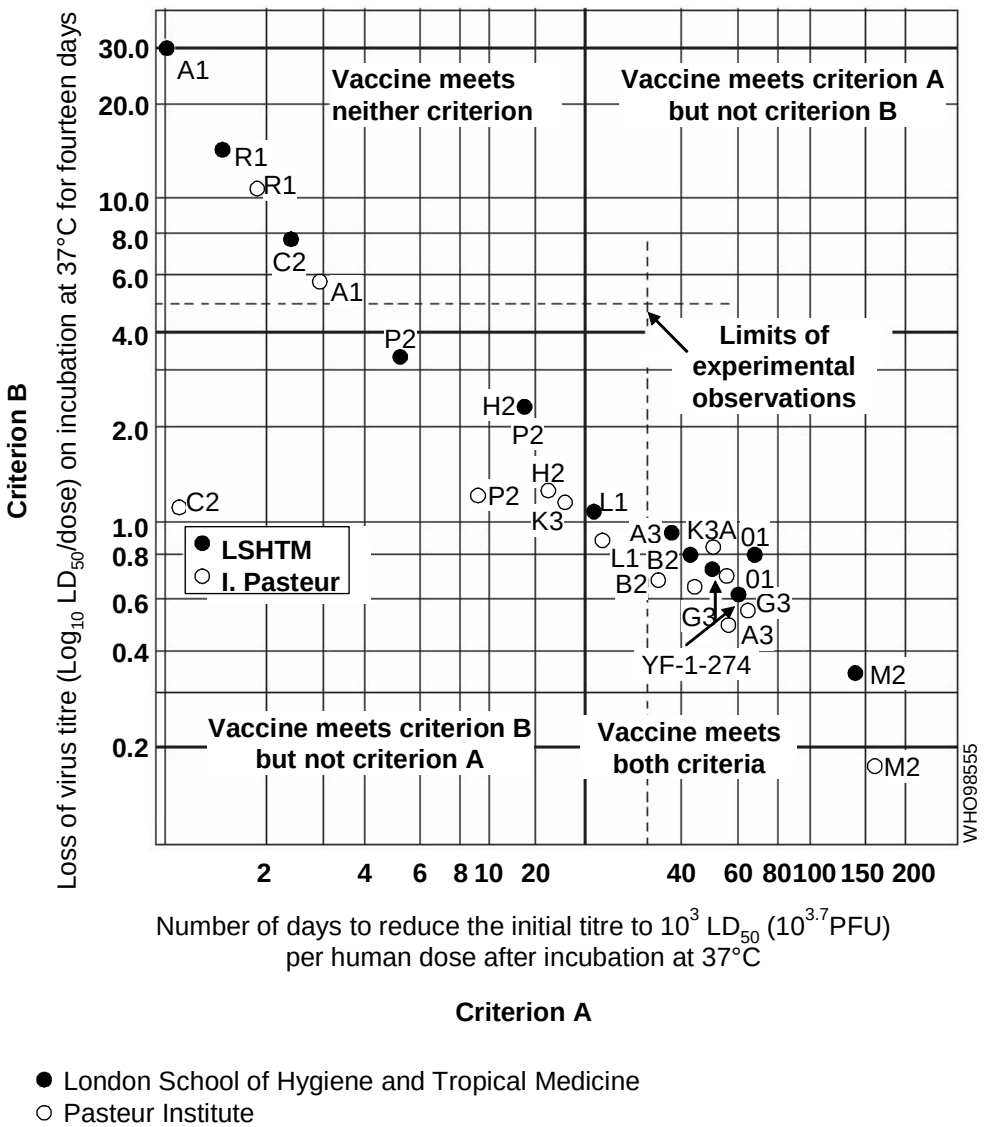
11.1 Stability of freeze-dried vaccine

Several manufacturers have introduced vaccines with enhanced stability. Media such as lactose, sorbitol, histidine and alanine have considerably improved the heat stability of lyophilized 17D yellow fever (YF) vaccine (*127*). These stabilized vaccines may successfully be used in different field conditions (*51, 118*).

Yellow fever vaccine can safely be stored at -20°C or +4°C for two years or more (*21, 109*). The estimated half life of the vaccine infectivity is 3 to 10 months at room temperature, from 10 to 20 days at 37°C, and about two days at 46°C (*21, 46*). The time required to reduce the initial titer of the vaccine to 1000 infective units is between 2.6 to 6.1 days at 46°C, 5.7 to 15.7 days at 37°C and 12.4 to 26 days at 31°C (*67*). In 1987, a study of 11 YF vaccines was sent to WHO by manufacturers. This showed a wide range of stability among vaccines (*184*). The number of days required to reduce the initial titer to 1000 infective units, when vaccines were kept at 37°C, ranged from one to five days in four vaccines, from 13 to 21 days in two vaccines, and from 38 to 146 days in five vaccines (Figure 7).Two products showed stability at 2-8ºC for 36 months, at 25ºC for nine months, and at 37ºC for 72 days.

In Figure 7, the vaccines which appear above the horizontal line indicating the limit for a loss of 1 $\log_{10}$ of virus underwent too high a titer loss. Likewise, the vaccines which appear to the left of the vertical line which indicates 14 days of incubation experienced too high a loss of potency. Vaccines which appear in the lower-right quadrant meet both criteria A and B of the WHO requirement for stability. This requirement stipulates that the vaccine should retain 1000 mouse LD50 or the equivalent in plaque-forming units (PFUs) per human dose (A), and that the mean titer loss should be less than 1 $\log_{10}$ after two weeks' incubation at 37°C (B) (*167*). This requirement is met by all prequalified YF vaccines (*174*).

Figure 7: Rating of 17D yellow fever vaccines according to proposed requirements for heat stability



Source: World Health Organization (*184*)

11.2 Stability of reconstituted vaccine

When kept at between 2°C and 8°C, the reconstituted vaccine retains its minimum immunizing dose (1000 infective units) for at least 10 days (*67*). However, when the reconstituted vaccine is exposed to elevated temperatures, it quickly deteriorates. At 37°C, 31°C and 27°C, the reconstituted vaccine lost 50% of its infectivity following 1.5, 3.1 and 4.9 hour exposures (*21, 67*). An exposure of reconstituted vaccine for one hour to 46°C resulted in a 0.5 log_{10} loss, and after a two-hour exposure, the loss of infectivity exceeded 1 log_{10} (*21*). Regardless of stability of a reconstituted vaccine, because of the risk of contamination, such products should be kept cold after reconstitution and discarded at the end of a 6-hour immunization session.

11.3 Summary

Lyophilized yellow fever vaccine can be safely stored at -20°C or +4°C for two years. Vaccines meeting the WHO stability guidelines show a minimum mouse potency titer (or an equivalent potency in PFU) of greater than 1000 units after exposure to 37ºC for 14 days, and their loss in potency during this exposure is less than 1 $\log_{10}$. As with the measles vaccine, yellow fever vaccine quickly deteriorates after reconstitution when it is exposed to elevated temperatures. Yellow fever vaccine should be quickly administered after reconstitution, maintained at 2-8°C, and discarded at the end of the session, not only to preserve potency, but to minimize risk of contamination of this lyophilized vaccine once reconstituted.

12. Poliomyelitis vaccines

Remarkable progress has been achieved over the past years towards global eradication of poliomyelitis. The oral poliomyelitis vaccine (OPV) has been the vaccine of choice for this campaign. Oral poliomyelitis vaccine is the least stable of the vaccines commonly used in national immunization programmes. It uses a live, attenuated virus which is unstable except when held at low temperatures. Current recommendations require that, for maintenance of potency, the vaccine must be stored and shipped at low temperatures (-20ºC). The vaccine's thermostablity has been improved through the use of stabilizers such as high concentrations of magnesium chloride or sugars in a well-buffered solution. These are systematically used to stabilize all OPV preparations.

12.1 Overall stability of poliomyelitis vaccine at elevated temperatures

The stability of trivalent poliomyelitis vaccine has usually been monitored by measuring the total content of live viruses of three serotypes (*126*). This practice may overlook changes in the type 2 and type 3 content following exposure to elevated temperatures (*8).* Little loss in virus titer has been observed after long-term storage of OPV at -20°C (*126*). Most manufacturers indicate that their OPV vaccines are potent if stored at -20°C or less until the expiry date indicated on the packing (usually two years).

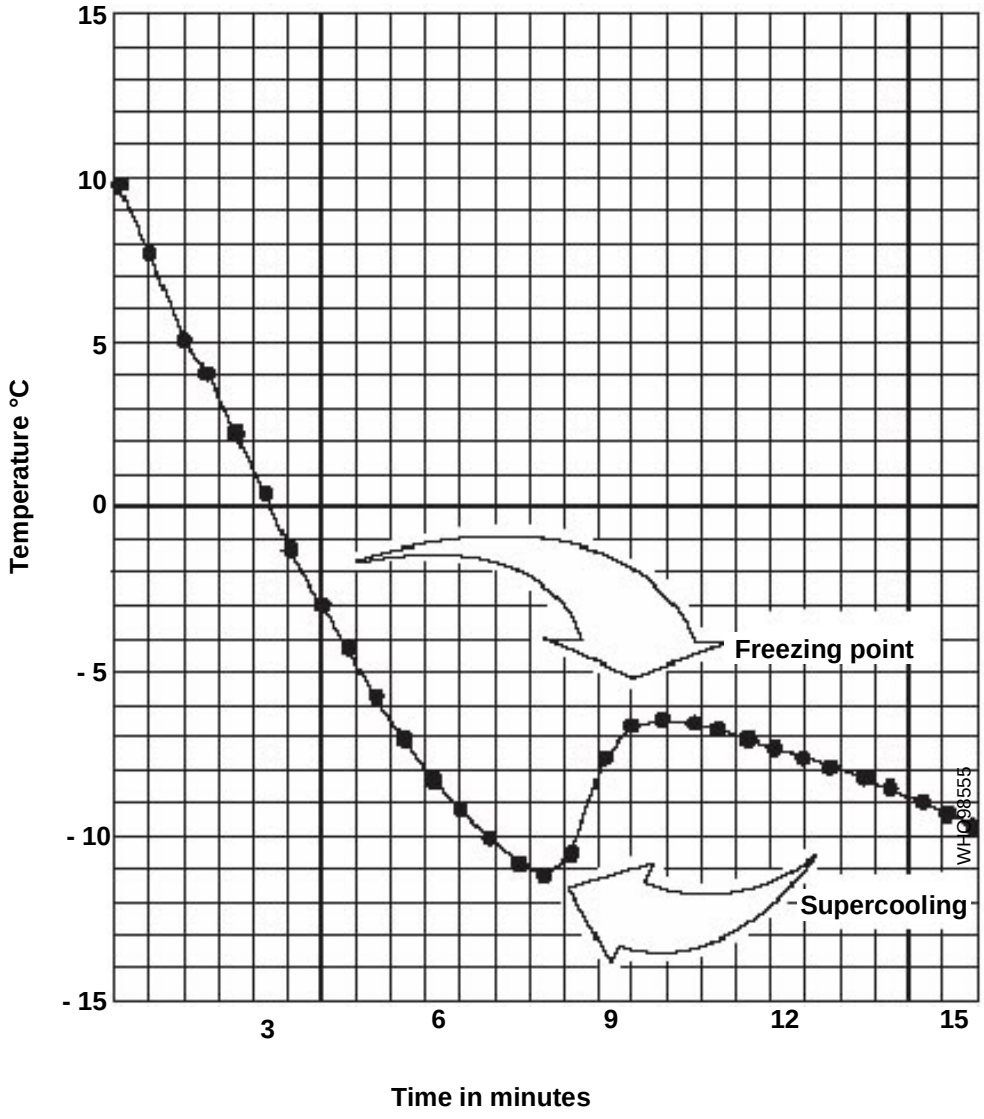
12.2 OPV at freezing temperatures

12.2.1 Freezing point of OPV

The presence of stabilizers in vaccine preparations lowers their freezing point. A study was carried out on trivalent OPV from five manufacturers to determine the freezing points of the products and the effects on their potency of up to 180 cycles of freezing and thawing (*43*).

When poliomyelitis vaccines are kept at -25°C they supercool rapidly to between -8°C and -16°C, while remaining in the liquid state. Their temperature then rises rapidly to about -7.5°C while solid freezing occurs. The temperature to which the vaccine rises is taken as the freezing point (Figure 8), which varies from -6.6°C to -8.1°C (*43*).

Figure 8: Temperature of trivalent poliomyelitis vaccines exposed to -25°C for 15 minutes



Source: World Health Organization (43)

12.2.2 Vaccine potency after repeated freezing-thawing cycles

Some studies have shown that there is no significant loss of virus titer in OPV subjected to up to 10 cycles of freezing and thawing (*13*, *42*, *126*). However, no details were given concerning the rapidity of freezing and thawing, the temperature to which samples were raised during each thawing, or the length of the intervals during which the vaccine was kept thawed. The total titers for trivalent vaccine were measured but no data were given for type-specific poliovirus sensitivity to freeze-thaw cycles. All these factors may influence the survival of virus particles during such cycles (*8*).

Vaccines subjected to 10, 90, and 180 freeze-thaw cycles (from -25°C to 2.5°C) had virus titer values for all three types which were not significantly different from those of control samples held at -25°C. There was no trend towards a decline in titer as the number of cycles increased (*43*). However, the maximum temperature did not exceed 2.1°C. Under field conditions, a break in the cold chain can result in vaccines reaching much higher temperatures. Consequently, these results are valid only for situations where the temperature of thawed vaccine remains in the range found in a refrigerator which is working properly.

Freezer compartments of refrigerators, which are sometimes used for the storage of OPV, operate at about -5°C. This is above the melting point of the vaccine, which may not, therefore, remain solid.

12.3 WHO requirements for thermostability

Each final lot of OPV must undergo the accelerated degradation test to confirm that its stability is satisfactory. Representative final containers of the vaccine have to be incubated at 37°C for 48 hours. The total virus content in both exposed and unexposed vials is determined concurrently with that of a trivalent reference preparation. The vaccine passes the test if the loss on exposure is not greater than a factor of $10_{0.5}$ infectious units per human dose. The national regulatory authorities are to specify the minimum virus titers per human dose (*165*).

12.4 Factors affecting OPV stability at high temperatures

The stability of OPV depends on several factors, including possible differences in heat sensitivity between viral types, the presence and nature of a stabilizer, the pH value of the vaccine, and the container in which the vaccine is stored.

12.4.1 Differences in heat sensitivity of viral types

The individual poliovirus types in the triple vaccine differ in their growth characteristics. Type 2, when administered in conjunction with types 1 and 3, has the most prolific growth during intestinal replication, followed by types 3 and 1 (*94, 133*). To compensate for these differences in growth rate, balanced formulations of trivalent vaccine were developed, usually containing types 1, 2 and 3 in the proportions of 10:1:3 (*165*). Further studies showed that changes in the ratio of these components might enhance the immunogenicity of OPV, particularly of the type 3 virus (*104, 105*).

12.4.2 Nature of stabilizer

The stabilizers most frequently used with attenuated polioviruses have been magnesium chloride and saccharose; buffers, milk and gelatin have also been used. Wallis and Melnick (*145*) reported that polioviruses were stabilized by the addition of cations to the suspending medium. In particular, the addition of 1 molar magnesium chloride (MgCl2) to attenuated poliovirus strains enabled vaccines to be stored at 4°C for three months or at 25°C for 25 days without significant loss of titer. Melnick et al. (*90*) found that vaccines stabilized with MgCl2 which were subjected to 30°C for 21 days elicited an antibody response equal to that of ordinary vaccine maintained in the frozen state and thawed just prior to administration.

Other studies showed that 35% to 50% sucrose was effective in stabilizing attenuated polioviruses. Magrath (*84*) concluded that both 1 M MgCl2 and 50% sucrose were effective stabilizing agents. To achieve maximum virus stability it was necessary to prevent the rise in pH that occurred as CO2 was lost from the container.

At present, most OPVs available on the market are stabilized with magnesium chloride. Recent studies appear to indicate that magnesium chloride is more effective than sucrose in increasing thermostability of OPVs. On exposure to 37°C for 8 days or to 25°C for 29 days the rate of potency loss in monovalent vaccines of all three types was higher in products stabilized with sucrose or buffer than in those stabilized with magnesium chloride.

A vaccine stabilized with magnesium chloride was more stable than one produced by the same manufacturer which was stabilized with sucrose at temperatures below 37°C (Table 9) (*107*). The better stabilizing effect of magnesium chloride has also been observed elsewhere (*95*), with data indicating a half-life of 92 years! It was concluded that the consistent use of magnesium chloride would help to increase the stability of OPV and to minimize reliance on the cold chain (*69*).

Table 9: Average losses of total virus content and half lives of oral poliomyelitis vaccines stabilized with sucrose and magnesium chloride and stored at various temperatures

Table 9: Average losses of total virus content and half lives of oral poliomyelitis vaccines stabilized with sucrose and magnesium chloride and stored at various temperatures

Storage temperature (°C)	Time unit	Sucrose		Magnesium chloride	
		Titre loss*	Half life	Titre loss*	Half life
4	Month	0.11	6 months	0.02	20 months
20-25	Day	0.03	12 days	0.01	23.1 days
37	Day	0.15	1.9 days	0.16	1.8 days
45	Day	-	-	0.61	0.6 days

* Per time unit shown, in $\log_{10}$ $CCID_{50.}$

Source: Peetermans JH, Colinet G (107).

In a WHO collaborative study on 46 samples, 12 stabilized with sucrose and the others with MgCl2, 83% of those stabilized with sucrose and 91% stabilized with MgCl2 lost less than 0.5 log10 CCID50 on incubation for 48 hours at 37°C. The mean loss was 0.34 log10 CCID50, irrespective of the stabilizer (*157*). Buffered sucrose can evidently also be an efficient stabilizer provided that the pH is carefully controlled.

Poliovirus can be also stabilized against heat inactivation by adding fatty acids and related compounds. Incubation of type 1 Sabin poliovirus with myristic acid at 45°C for 30 minutes caused a 19% reduction in infectivity, while incubation without this fatty acid resulted in a 99% loss of infectivity (*36*). Thermal stabilization was also observed when poliovirus was incubated with hexanoic, octanoic or palmitic acids.

The presence of these stabilizers during heating may prevent conformational changes in the capsid which render the virus non-infective.

Heavy water, deuterium oxide (D2O), also has a stabilizing effect on polioviruses. With its strong hydrogen bonds it can protect protein against denaturing and enhance the thermostability of poliovirus strains. The infectivity of three D2O- and MgCl2-treated OPV strains exposed to 37°C for seven days remained within the limit of requirements, i.e., they did not lose more than 0.5 log10 CCID50 (*189*). Despite the dramatic increase in the heat stability of OPV when heavy water was substituted for water, this avenue of research has not been pursued further, mainly for the following reasons:

- the available OPVs are sufficiently heat-stable to achieve their purpose of eradicating poliomyelitis;
- there would be disadvantages in licensing and introducing a new OPV while poliomyelitis eradication is in progress;
- VVMs are now used to monitor heat exposure in individual vials of OPV.

12.5 Inactivated poliomyelitis vaccine

The capacity of poliovirus to produce neutralizing antibodies is destroyed by heat treatment, freeze-drying and the addition of merthiolate (thiomersal). As mentioned previously, the poliovirus component of a quadruple DTP-inactivated polio vaccine was not stable when merthiolate was used as a preservative. Beale and Ungar (*12*) demonstrated a rapid fall in potency of the poliovirus antigen in a quadruple vaccine preserved with merthiolate and sodium edetate and stored at 4°C. Another lot of quadruple vaccine without merthiolate but with half the amount of sodium edentate was stable for one year. These observations were recently confirmed with the high potency inactivated poliomyelitis vaccine (eIPV), which was combined with DTP vaccine. Storage of eIPV at 4°C in the presence of merthiolate reduces the potency of type 1 poliovirus antigen to undetectable levels after four to six months. Type 2 and type 3 antigens are less markedly affected by exposure to merthiolate for eight months at 4°C (*120*). The incompatibility of eIPV and DTP vaccine preserved with merthiolate requires further study.

It appears that there are differences in heat stability between various inactivated poliovirus types, with type 1 being the most vulnerable. In the absence of preservative the type 1 component of trivalent IPV deteriorates slowly after storage for two years at 4°C, while the two other types remain potent for 20 years. The D-antigen content for type 1 drops significantly after 20 days at 24°C and is undetectable after exposure to 32°C for the same period; no significant changes in D-antigen are observed for type 2 at either of these temperatures; type 3 remains stable for 20 days at 24°C but the D-antigen content drops significantly at 32°C (*96*).

All three types of IPV show satisfactory retention of potency when incorporated into combined vaccines and stored at 4°C for periods ranging from one year to over four years. These observations were made on DT-polio vaccine preserved with 2-phenoxy-ethanol and adsorbed onto aluminum hydroxide (*96*), and on aluminum phosphate adsorbed DTP-poliomyelitis vaccine without preservative or with phenoxyethanol and formaldehyde as preservatives (*141*). Longer storage resulted in a decline in antigenicity, especially of the type 1 component (*96*).

Plotkin and Vidor summarize data showing that IPV is stable for 4 years at 4ºC and one month at 25ºC (*111*). At 37ºC there is a significant loss of potency of the type 1 component after 1-2 days and of types 2 and 3 after 2 weeks (*120, 143*). Freezing diminishes potency of IPV, related to loss of the D-antigen structure (*143*).

At 37°C the D-antigen content of the poliomyelitis component of a quadruple vaccine decreases during storage but most of the potency remains after eight weeks. Type 3 seems to be the most stable component (*141*).

12.6 Summary

OPV as supplied by most manufacturers is stable for an extended period at -20°C, for over six months at 2 to 8°C, and for over 48 hours at 37°C. VVMs help to deal with the limitations of this vaccine in respect of thermostability.

IPV is quite stable alone or when combined with other components at refrigerator temperatures (at least two years), and relatively stable at temperatures of 25ºC and 37ºC (up to one month and several days, respectively, but is rapidly inactivated by freezing or exposure to mercury compounds such as thiomersal.

Part III: Analysis of vaccine stability – other vaccines

13. Rotavirus vaccine

Previously a rotavirus vaccine (Rotashield) has been licensed in the US for use in infants, but its use was stopped because of association with reports of intussusception (*27*). Rotashield was stable for 24 months between 20-25° in the lyophilized state and for 60 minutes at room temperature or 4 hours in the refrigerator after reconstitution (*24*). An additional vaccine, Rotarix, has been licensed in Mexico and several other countries, and has been proposed for licensing in many others. It is available as a live attenuated product in a lyophilized format. Rotarix can be frozen in lyophilized format. Manufacturer's data gives two years of stability at -20°C without any potency loss. The stability data of the lyophilized form is confirmed for 1 week at 37°C (stress assay). Manufacturer's data also suggest that at 2°C to 8°C storage there is no potency loss at all after three years (114). A second rotavirus vaccine (Rotateq) was licensed in the US in February 2003 for use in infants. Manufacturer's data gives two years of stability at 2°C to 8°C storage. According to the manufacturer's product insert (*http://www.merck.com/product/usa/pi_circulars/r/products_r.html*), it can be stored and transported at 2-8°C, and should be protected from light. One additional rotavirus vaccine is licensed in China, made from a lamb strain of rotavirus. We were not able to find data on its stability.

14. Japanese encephalitis vaccine

At present, two types of formalin-inactivated Japanese encephalitis (JE) vaccines are in use (*59*). One, derived from mouse brain, is used in India, Japan, the Republic of Korea, Thailand, and other countries. The other is derived from primary hamster kidney cell culture and is used in China. The inactivated mouse brain derived Biken vaccine retains potency after storage in lyophilized form at 4° C for one year, at 22ºC for 28 weeks, and at 37ºC for four weeks (*135*). A freeze-dried JE vaccine produced in India is stable, undergoing falls in potency of only 4.7% in 52 weeks at 4°C and of 8.7% in 28 weeks at 22°C. Degradation is more rapid at higher temperatures: potency declines by 14% and 24% during 18 weeks at 37°C and 40°C respectively (*55*). After reconstitution the vaccine is still stable at 22°C. There is a 1% drop after two weeks, and a fairly rapid deterioration in potency in four weeks at 37°C and 40°C (*55*). We were not able to find data on the Chinese inactivated vaccine.

A live attenuated vaccine derived from the SA-14-14-2 strain and grown in primary hamster kidney cells is produed in China (*59*). This vaccine is stable at 37ºC for seven to 10 days, at room temperature for four months, and at 2-8ºC for 1.5 years. After reconstitution, it is stable for two hours at 23ºC (*146*). At present this vaccine has limited availability on the international market.

15. Typhoid vaccines

In the past, parenteral killed whole cell typhoid vaccines have been widely used but they frequently caused local pain and swelling, fever, headache, and malaise. In the most recent years, two typhoid vaccines have been widely used: parenteral Vi polysaccharide-carrier protein conjugated vaccine and attenuated *Salmonella typhi* strains used as live oral vaccines (*81*).

The purified Vi polysaccharide vaccine is highly stable and does not require a cold chain even in tropical conditions. It is a liquid in phenolic isotonic buffer, and is stable to six months at 37ºC and for two years at 22ºC. However, it should not be frozen.

The live oral vaccine contains the Ty21, a mutant of *S. typhi*, and should be stored at + 4°C. It is available as an enteric-coated capsule that is reconstituted in buffer. The shelf life of the lyophilized vaccine is dependent on residual moisture content and maintenance of the cold chain. Study of errors in following prescribing information showed that about half of them were related to incorrect storage temperature (*31*). Vaccine failures in Swiss travelers have been associated with vaccine which was not kept in a refrigerated state (*62*). Prolonged storage at room temperature resulted in progressively lower viable counts over time, although all tested lots evaluated after storage for seven days at 20°C to 25°C met potency requirements. Three lots of the vaccine stored at 37°C for 12 hours also maintained potency (*30-32*).

16. Cholera vaccines

Although parenteral killed cholera vaccines are not recommended by WHO to any persons of any age, they are still available commercially in many countries. Recently, a killed oral whole cell vaccine combined with B subunit (WC/BS vaccine) has been developed in an attempt to stimulate local intestinal mucosal immune responses in a manner similar to that induced with natural exposure (*119*). The storage requirements for oral killed vaccine are similar to these needed for the former parenteral vaccines (the vaccine is stable for three years when kept in the refrigerator at 2-8°C). This vaccine comes with buffer to be dissolved in water and taken by mouth. It should be kept refrigerated but not frozen (*119*)

Another recently developed vaccine is a live oral CVD103 HgR vaccine. Because the vaccine strain is live, the viability of bacteria must be preserved while in storage and an effective cold chain is likely to be needed. The bacteria must be protected from stomach acids with buffer, and the product must be administered immediately after reconstitution (*119*).

17. Hepatitis A vaccine

Harvests of hepatitis A virus, multiplied in cell culture, are clarified, purified and concentrated and then inactivated by formaldehyde. Aluminum hydroxide or aluminum phosphate is used as adjuvant in the case of three of the products (Merck, GSK, and Sanofi-Pasteur); the Berna vaccine has liposome adjuvant (*14*). The aluminum adjuvanted vaccines should not be frozen. Both the Merck and the GSK products retain potency for two years at 2-8º C (*14, 106, 113*). ADTs performed on GSK vaccine lots at release and after storage for 15 months in a refrigerator indicate no loss of immunogenicity at 37°C for up to three weeks (*106*). The reactogenicity and immunogenicity of Havrix (the GSK product) was stable after 37ºC for one week (*25, 113, 185, 186*). The Merck product, VAQTA, did not differ in its stability profile at the recommended storage temperature when stored at 37ºC for 12 months (*25, 113*).

18. Rabies vaccine

The solution to the problem of rabies vaccine safety is in the development of vaccines prepared from rabies virus grown in tissue culture free from neuronal tissue (*110*). Since 1976, use of human diploid cell vaccine (HDCV) has become general for pre- and post-exposure immunization of humans. HDCV vaccine evokes much better immune responses than did vaccines prepared from virus propagated in embryonated duck eggs, or vaccines prepared from suckling mouse brain (Fuenzalida vaccine) or adult sheep or rabbit brains (Semple vaccine).

HDCV in its lyophilized form is a very stable vaccine; it retains its potency for at least 3.5 years at temperatures between 2°C and 8°C (*26*) and for one month at 37°C (*99, 138*). Another HDCV vaccine was stable for at least 18 months at -20°C and + 4°C, and withstood exposure to 37°C and 60°C for three months (*85*). A third HDCV (*122*), available in liquid form, meets similar stability criteria.

Human diploid cell rabies vaccine is difficult and expensive to produce. There have been intensive efforts worldwide to produce vaccines at a low cost that can meet or improve on the levels of safety and efficacy achieved with HDCV (*110*). The new vaccines include: the purified chicken embryo cell vaccine (PCEV) developed By Chiron Behring in Germany (formerly Behringwerke), which was stable for three months at 37°C (*11*): primary Syrian hamster kidney cell culture vaccine, developed in the Poliomyelitis Institute in Moscow, Russian Federation and used in China; the Vero cell line vaccine developed by Sanofi-Pasteur, France; and purified duck embryo vaccine developed in the Swiss Serum and Vaccine Institute in Switzerland. All are likely to have much better stability than the former vaccines prepared on neural tissues.

Cell culture rabies vaccines which are currently prequalified by WHO and therefore meet WHO potency and stability requirements (*166*) include the products of Chiron Behring, Germany, and Chiron Behring, India (both PCEV), and the Vero cell vaccine developed by Sanofi-Pasteur, France.

19. Influenza vaccines

Influenza vaccines can be either inactivated or live-attenuated. There are several types of inactivated influenza vaccines, varying according to production source (egg; cell-culture), content (split; subunit; whole virion), production virus (classical reassortment; recombinant), adjuvants (e.g. MF59), attenuating agents (e.g. formaline, Beta-propiolactone) or type of purification (centrifugation; concentration). Stability depends on careful attention to specific formulation, addition of stabilizers such as gelatin or polysorbate, compatibility of the product with container and closure and preparative treatments needed to reduce adsorption or interaction with container, pH etc (*48*). Seasonal influenza vaccines are valid for one year only because of the need to adapt the vaccine strain to the circulating field virus. Most products are stable for one year at 2 - 8° C. The potency of the individual virus vaccine components can decline at varying rates, as seen, for example, with an A H3N2 component that lost potency faster that expected (*112).*

Live attenuated influenza vaccines have been used for several decades in Russia and have recently been developed in the USA(*16*), for intranasal application. It contains sucrose-phosphate-glutamate stabilizer and was developed from a cold-adapted strain of influenza. It must be stored frozen (-15°C to -25°C), and thawed for up to 60 hours at +2°C to +8°C before use, but it should not be refrozen (*116*). Because temperature cycling could affect product stability, it should be stored in a frost-free freezer (*123*). A refrigerator stable formulation (to be kept at +2°C to +8°C) is in development.

20. Varicella vaccine

There are three producers of varicella vaccines made on human cells : Merck, GSK, and Biken (distributed by Sanofi-Pasteur). The vaccine is a cell-free virion preparation that generally contains sucrose and buffer salts, and is lyophilized (*52*). The lyophilized form can be stored at refrigerator temperature for 1.5 years or more, but manufacturers suggest it is better stored frozen. It should not be refrozen (*115*).

Part IV: Final conclusions

21. Future demands on the cold chain

Technologies for vaccine delivery in the 21st century document published by WHO, in 2000 (*171)* provides a vision of the evolution of the cold chain in the 21st century. The factors that will influence how vaccines are stored will depend on new strategies, new vaccines, and new tools. For example, for disease control, there is more emphasis on delivering vaccines by campaign, which will change the concepts of their storage. In addition, to increase coverage, in areas with far-flung populations, more emphasis is being placed on outreach immunizations, which will also place special demands on the traditional cold chain.

New vaccines are being developed, and the time is now for immunization managers to communicate with vaccine developers to ensure that the most thermostable options are explored. PATH is actively investigating vaccine stabilization technologies (*103*). They are facilitating research on the technical and commercial viability of thermostable vaccines in collaboration with commercial partners. Technical research is focused on sugar-glassification and spray-drying based technology applied to both core and new EPI vaccines. Feasibility studies are underway with measles and hepatitis B vaccines. The next round of technologies and antigens to be studies will be influenced by the results of these studies. Ideally, new thermostable vaccines would be regulated for shelf life storage at temperate or tropical room temperatures. The availability of thermostable vaccines will improve immunization effectiveness through reduction of potency loss due to heat or freeze damage and reduce cold chain costs and logistical requirements.

Finally, new tools have been developed and are being used which will allow flexibility in vaccine management. These include the VVMs, discussed above, as well as better tools to monitor vaccine freezing in the field.

22. Implications for the field

The stability of vaccines varies considerably. They can be ranked by their resistance to storage at elevated temperatures, with diphtheria and tetanus toxoids and hepatitis B vaccine showing the highest thermostability, freeze-dried measles, yellow fever and BCG vaccines occupying the middle position and oral poliomyelitis vaccine being the most fragile. Reconstituted vaccines against measles, yellow fever and tuberculosis (BCG) are unstable vaccines and are subject to bacterial contamination; they should be used as soon as possible after reconstitution, be kept in a ice bath during the immunization session and should be discarded at the end of the session.

The data presented show that some vaccines can withstand a long period of exposure without a significant loss of potency. The high resistance of tetanus toxoid and hepatitis B vaccine to heat has spurred studies on the use of these vaccines without refrigeration. They retain sufficient potency during short-term exposure to heat when used in outreach programmes for the immunization of women of childbearing age against tetanus or immunization of children against hepatitis in areas where the cold chain cannot be maintained. As mentioned above, a study performed in Indonesia with hepatitis B and tetanus toxoid vaccines in prefilled, single-use injection devices, "UniJects" (*132*), suggests that such a device represents a potential cost-effective strategy for outreach immunization. Moreover, used as described in Indonesia, it could also eliminate the problem of these vaccines freezing during transport. This is an example of the kind of programmatic impact that can be achieved by understanding and taking advantage of the stability of vaccines in use.

Despite the heat stability of some products, other vaccines are more sensitive to changes in temperature. For these products, each exposure to elevated temperature results in some degradation of the vaccine, even if the remaining potency is still above the level considered to be the minimal immunizing potency. Furthermore, each exposure to ambient temperature has a cumulative impact on vaccine potency. Vaccines in peripheral health units could not withstand the temperatures mentioned above if their potency had already been compromised by previous breaks in the cold chain. The data presented in this document may be useful for those involved in immunization activities at central and provincial levels who have to make decisions about vaccines exposed to elevated temperatures.

All vaccines should be routinely stored at the temperatures recommended by manufacturers and national immunization programmes. The cold chain remains a highly vulnerable point for all immunization programmes. In all countries, systems of refrigeration, temperature-monitoring and record-keeping are required to make sure that each vial of vaccine is maintained under appropriate conditions and that it is used before the expiry date shown on the label.

These changes will require behavior modifications and more knowledge on the part of immunization managers, including:

- Better understanding of the characteristics of the vaccine products they are using;
- Better vaccine management practices;
- A cold chain which is tailored to the particular needs of the situation;
- More attention to the problem of freezing vaccines;
- More impact of loss of products due to freezing or excessive heat exposure, as the new vaccines will be more expensive.

A summary of information on the stability of vaccines stored at various temperatures is presented in the following summary tables.

Table 9: Stability of vaccines commonly used in national immunization programmes

Vaccine[4]	Storage temperature, ºC				
	2-8	20-25	37	>45	Freezing
Tetanus and diphtheria toxoids, monovalent or components of combined vaccines[5]	Stable for >3 years	Stable for months	Stable for months	Unstable above 55ºC	Unstable: do not freeze
Hepatitis B vaccine[2]	Stable for >4 years	Stable for months	Stable for weeks	At 45ºC, stable for days	Unstable; do not freeze
Measles, mumps, rubella vaccines[1]	Stable for 2 years	Stable for at least one month	Stable for at least one week	Unstable	Stable
Yellow fever[1]	Stable for >2 years	Stable for months	Stable for two weeks	Unstable	Stable
Pertussis vaccine[2]	Stable for 18-24 months	Stable for 2 weeks	Stable for one week	10% or more loss of potency per day	Unstable; do not freeze
BCG vaccine[1]	Stable for 1-2 years	Stable for months	Loss of no more than 20% after one month	Unstable	Stable
Oral poliovirus vaccine	Stable for up to 1 year	Stable for weeks	Stable for 2 days	Unstable	Stable
Inactivated poliovirus vaccine[2]	Stable for 1-4 years	Stable for weeks	Stable for weeks	Little data available	Unstable; do not freeze
Polysaccharide vaccines (meningitis, pneumococcal)[1]	Stable for 2 years	Stable for weeks to months	?	?	Unstable; Do not freeze
Conjugate polysaccharide vaccines (meningitis, Hib, pneumococcal)[1]	Stable for > 2 years	Stable for > 2 years	May be unstable, depends on presentation	Unstable	If in combination with aluminum adjuvanted vaccine, do not freeze

[4] *In lyophilized form for measles-mumps-rubella, BCG, yellow fever, some polysaccharide vaccines; other vaccines in liquid form. Reconstituted vaccines are not included as they must be discarded at the end of a session, as they have little or no stabilizers and so risk contamination, as well as being less stable.*

[5] *Aluminum stabilized, or may be presented in this way.*

Table 10: Stability of other bacterial and viral vaccines

Vaccine[4]	Storage temperature, ºC				
	2-8	20-25	37	>45	Freezing
Hepatitis A vaccine[6]	Stable for 2 years	?	Stable for 1-3 weeks or more	?	Unstable: do not freeze
Human diploid cell rabies vaccine[2]	Stable for 3-5 years	Stable for 18 months	Stable for 4 weeks	Stable for several weeks	Stable
Japanese encephalitis B vaccine, inactivated[2]	Stable for 1 year	Stable for 28 weeks	Stable for 4 weeks	Unstable	Stable
Japanese encephalitis B vaccine, live[7]	Stable for 1.5 years	Stable for 4 months	Stable for 7-10 days	Unstable	Stable
Inactivated cholera and typhoid vaccines[1]	Stable for > 2 years	Stable for years	Stable for 6 months	No data available	Unstable; do not freeze
Live attenuated cholera and typhoid vaccines[2]	Stable for 1 year	Stable for 7 days	Stable for 12 hours	Unstable	Stable
Influenza, inactivated	Stable for up to 1 year	?	?	?	?
Influenza, live[8]	Stable for 60 hours	Unstable	Unstable	Unstable	Store frozen, do not refreeze
Varicella vaccine[3]	Stable for 1.5 years	?	?	?	May store frozen, do not refreeze
Rotavirus vaccine[2]	Stable for > 2 years	Stable for 2 years	?	?	Stable

[6] *Aluminum adjuvanted*
[7] *Lyophilized*
[8] *Frozen*

Part V: References

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Annex 1:
Shake test learning guide

The shake test learning guide is developed for use in the training courses offered by the GTN/VM. However, it may be used effectively as part of other training courses or separately for on the job training activities.

The learning guide provides the steps of an accepted, standard and correct way of performing a shake test, evaluating the results and suggests the correct actions for different outcomes. It is a decision making tool to decide whether a suspect vaccine vial is damaged by freezing.

This learning guide is designed to be used during the demonstration and coaching sessions of a training program. The trainee is also encouraged to use it while practicing by her/himself.

1. Using the learning guide during demonstration

Teaching of a new skill should start with demonstration. A demonstration is basically showing how a skill is performed.

The trainer must first of all, make sure that s/he performs the skill precisely as outlined in the learning guide. All the steps are there for a reason, and none should be skipped or modified. The trainer must be proficient in performing the shake test. This issue cannot be overemphasized.

A demonstration should be as close to the real thing as possible. Therefore, having real vaccine vials (frozen vial for control, frozen and non-frozen vials for test) during the training is advised.

The trainer distributes the learning guides to participants prior to demonstration and goes through each step making sure all is clear about the instructions. The trainer answers questions about the learning guide.

The next step is to show how to do the shake test. Following the proper demonstration guidelines, the trainer demonstrates the shake test by actually doing it. At this point, the participants should be following the trainer and their learning guides at the same time. They are free to ask questions at all times during demonstration and coaching.

2. Using the learning guide during coaching

Coaching is a one-to-one activity between a trainer and a trainee on learning to perform a specific skill. During this activity, the trainee performs the shake test, and the trainer watches the trainee to provide encouragement, support and feed-back. The trainee uses the learning guide during this session. The trainer should emphasize the importance of performing the shake test exactly as it is written on the learning guide.

Since both demonstration and coaching are psycho-motor skills themselves, they also have their own learning guides outlining the necessary steps in performing them. A trainer should be proficient in performing demonstration and coaching.

3. Using the learning guide during self-practice

A trainee is encouraged to use the learning guide during self-practice. A partner may help the trainee, providing him/her with specific feed-back based on the steps of the learning guide. Self practice with or without a partner is a highly desirable situation which allows the trainee to pace his/her own learning experience, which in turn reduces the anxiety associated with learning a new skill, thus actually facilitating learning.

4. Using the learning guide during final assessment

This learning guide can be used to assess whether the trainee is in fact, competent to perform a shake test. Using the same tool for assessment and for learning, reduces performance anxiety on behalf of the trainee.

The structure of the learning guide

Performance assessment scale

The scale is used to mark whether each step is performed to a satisfactory level. We expect to see no “1”s to declare the trainee as “competent to perform the shake test” in the final assessment.

“Practice No.” columns

Each numbered column indicates one practice session of the trainee. When practicing with a partner, the partner would write the appropriate number from the assessment scale to each cell in that column. It is assumed that three practices would be sufficient to learn to perform the shake test. If the trainee feels s/he should practice more, s/he should be provided with more blank learning guides.

Shake test learning guide

Name of the trainee: _____

Performance assessment scale:

1) *Insufficient*: Trainee performs the step incorrectly, or not in the right order or skips the step altogether.
2) *Competent*: Trainee performs the step correctly and in the right order but either misses some points or needs to be reminded and encouraged by the trainer.
3) *Proficient*: Trainee performs the step correctly, in the right order, and without hesitating.

NOTE: The test procedure described below should be repeated with all suspect batches. In the case of international arrivals, the shake test should be conducted on a random sample of vaccine. However, if there is more than one lot in the shipment, the random sample must include a vial taken from each and every lot.		**Practice no.**		
1. Take a vial of vaccine of the same type and batch number as the vaccine you want to test, and made by the same manufacturer.				
2. Clearly mark the vial as "FROZEN."				
3. Freeze the vial at –20°C overnight, until the contents are completely solid.				
4. Let it thaw. Do **NOT** heat it!				
5. Take your "TEST" vial from the batch that you suspect has been frozen.				
6. Hold the "FROZEN" vial and the "TEST" vial together in one hand.				
7. Shake both vials vigorously for 10-15 seconds.				
8. Place both vials on a flat surface side-by-side and start continuous observation of the vials until test is finished. *(NOTE: If the vials have large labels, which conceal the vial contents, turn both vials upside down and observe sedimentation in the neck of the vial.)*				
9. Use an adequate source of light to compare the sedimentation rates between vials.				
	IF,			
10. The TEST vial sediments slower than the FROZEN vial, **THEN,**	10. Sedimentation is similar in both vials **OR** The TEST vial sediments faster than the FROZEN vial **THEN,**			
11. Use the vaccine batch.	11. Vaccine damaged; discard all affected vaccine.			
	12. Notify your supervisor.			
	13. Fill in the Loss/Adjustment Form.			

The figure below displays visually the difference between frozen control and (non-frozen) test vial.

Seeing the difference in sedimentation rates during a shake test

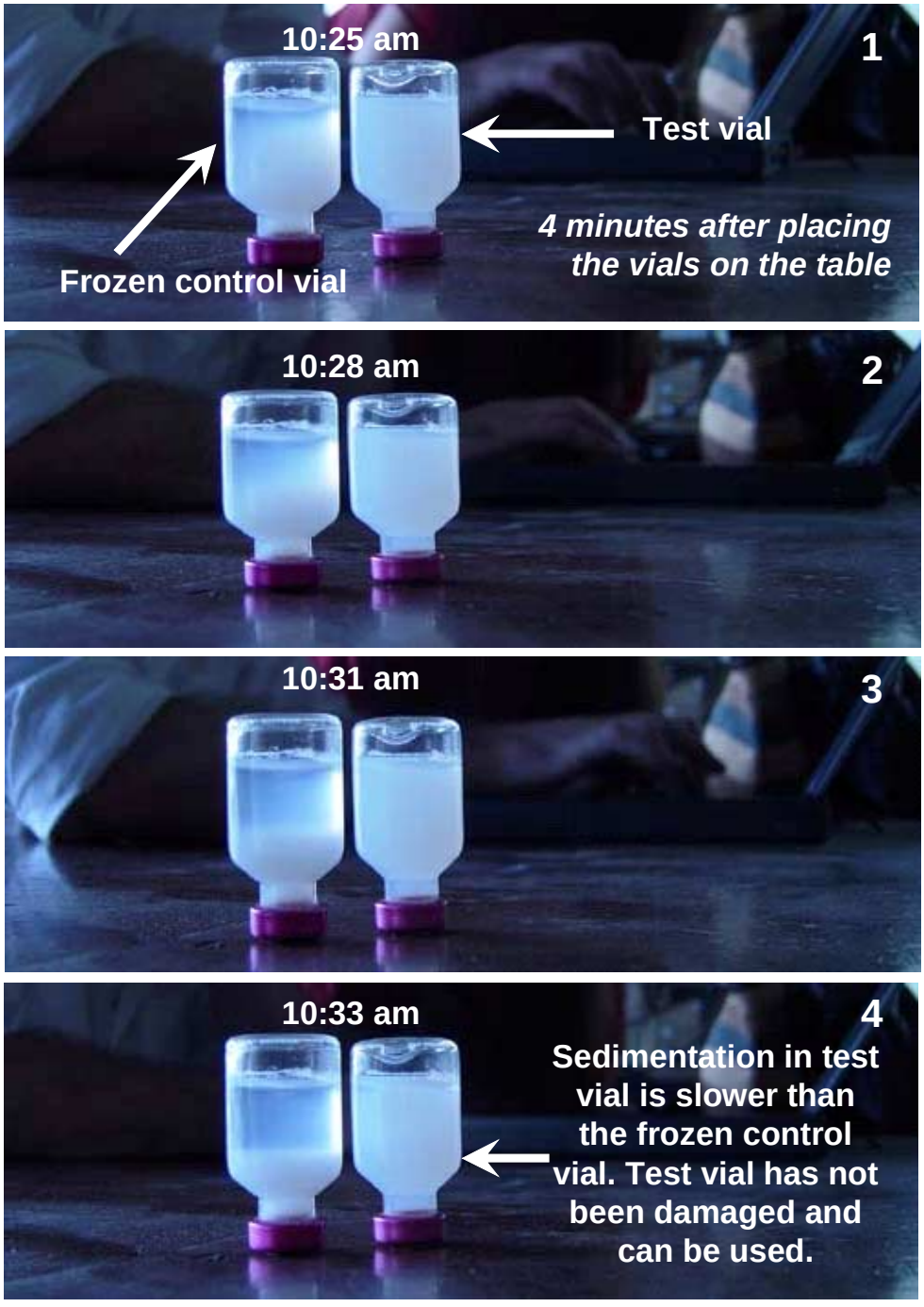

Photo: U. Kartoglu (WHO)

The World Health Organization has managed cooperation with its Member States and provided technical support in the field of vaccine-preventable diseases since 1975. In 2003, the office carrying out this function was renamed the WHO Department of Immunization, Vaccines and Biologicals.

The Department's goal is the achievement of a world in which all people at risk are protected against vaccine-preventable diseases. Work towards this goal can be visualized as occurring along a continuum. The range of activities spans from research, development and evaluation of vaccines to implementation and evaluation of immunization programmes in countries.

WHO facilitates and coordinates research and development on new vaccines and immunization-related technologies for viral, bacterial and parasitic diseases. Existing life-saving vaccines are further improved and new vaccines targeted at public health crises, such as HIV/AIDS and SARS, are discovered and tested *(Initiative for Vaccine Research).*

The quality and safety of vaccines and other biological medicines is ensured through the development and establishment of global norms and standards *(Quality Assurance and Safety of Biologicals).*

The evaluation of the impact of vaccine-preventable diseases informs decisions to introduce new vaccines. Optimal strategies and activities for reducing morbidity and mortality through the use of vaccines are implemented *(Vaccine Assessment and Monitoring).*

Efforts are directed towards reducing financial and technical barriers to the introduction of new and established vaccines and immunization-related technologies *(Access to Technologies).*

Under the guidance of its Member States, WHO, in conjunction with outside world experts, develops and promotes policies and strategies to maximize the use and delivery of vaccines of public health importance. Countries are supported so that they acquire the technical and managerial skills, competence and infrastructure needed to achieve disease control and/or elimination and eradication objectives *(Expanded Programme on Immunization).*

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