Evidence-based biosafety: a review of the effectiveness of microbiological containment measures

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Inhoudsopgave / Table of Contents

Management samenvatting ten behoeve van beleidsdoeleinden

In opdracht van de Commissie Genetische Modificatie (COGEM) is in deze studie nagegaan in hoeverre de huidige praktijk en regelgeving ten aanzien van het veilig werken met genetisch gemodificeerde micro-organismen (GMOs) gebaseerd is op wetenschappelijk bewijs, ofwel in hoeverre er sprake is van – in goed Nederlands – een "evidence-based biosafety" praktijk. Meer in het bijzonder waren de vraagstellingen van het onderzoek:

- Op welke wetenschappelijke verkregen gegevens berust de classificatie in ML-I tot en met ML-IV werkvoorschriften en inperkingsmaatregelen voor werkzaamheden met GMO's?
- Zijn er experimentele of observationele gegevens die de effectiviteit van werkvoorschriften en inperkingsmaatregelen onderbouwen?

Maatschappelijke ontwikkelingen

Er is een duidelijke trend zichtbaar om handelingen en beleid zoveel mogelijk te baseren op wetenschappelijk verkregen bewijs. Deze trend is vooral sterk ontwikkeld in de geneeskunde en staat daar bekend als "evidence-based" geneeskunde. Deze ontwikkeling strekt zich inmiddels uit over vele disciplines en beleidsterreinen. In de geneeskunde is inmiddels veel ervaring opgedaan. Zo wordt er hiërarchisch onderscheid gemaakt in de kwaliteit van het bewijs ("evidence") dat aan bepaalde handelingen ten grondslag ligt. Aan de mening van experts wordt hierbij een geringere betekenis toegekend dan aan systematische meta-analyses van peer-reviewed artikelen van gerandomiseerde dubbel-blinde klinische studies.

Deze ontwikkeling is voor de COGEM aanleiding geweest om na te gaan in hoeverre de huidige praktijk en regelgeving ten aanzien van het veilig werken met GMOs "evidence-based" is.

Historische ontwikkelingen

Vanaf het midden van de vorige eeuw is er gestaag gewerkt aan het opstellen van richtlijnen voor het veilig werken met micro-organismen onder leiding van instanties als de National Institutes of Health (NIH), de Centers for Disease Control and Prevention (CDC), de World Health Organization (WHO), de Europese Unie (EU) en de Nederlandse Vereniging voor Microbiologie (NVvM). In Nederland zijn naast het verantwoordelijke Ministerie voor Volkshuisvesting, Ruimtelijke Ordening en Milieu (VROM), het bureau GGO, de COGEM en de vereniging BVF-platform betrokken bij de implementatie van nationale wet- en regelgeving met betrekking tot GMOs. Deze activiteiten hebben geleid tot de huidige regelgevingpraktijk, waarvan gezegd kan

worden dat deze grotendeels berust op opgedane ervaringen, het oordeel van experts, en gezond verstand. Het doel van de wetgeving is bescherming van mens en milieu. Hoewel de voorschriften in het algemeen duidelijk zijn, zijn de doelstellingen die regelgevers met specifieke voorschriften beogen niet altijd expliciet vermeld, wat hun evaluatie in de weg kan staan. Een belangrijke constatering is dat de procedures rond GMOs afgeleid en in belangrijke mate identiek zijn aan die rond niet-GMOs, uiteraard omdat de risico's van het werken met GMOs en niet-GMOs, en de maatregelen om die in te perken, in belangrijke mate overeenkomen. In het rapport worden daarom beide in beschouwing genomen.

Principes en methodes om biosafety te bewerkstelligen

De huidige regelgeving en biosafety praktijk lijken te berusten op enkele principes en methodes die als volgt gegroepeerd kunnen worden:

- Risico assessment; dit is de eerste en centrale stap die bestaat uit het identificeren van mogelijke risico's voor mens en milieu, het inschatten van de kansen dat die risico's optreden, het inschatten van hun gevolgen, en het toekennen van maatregelen om die risico's te beheersen. Bij onzekerheid over de mate van risico, wordt het voorzorgprincipe gehanteerd.
- Biologisch inperken; waar mogelijk kunnen de risico's voor medewerkers en het milieu beperkt worden door het gebruik van micro-organismen, veelal GMOs, die minder virulent zijn, minder goed of niet repliceren, minder goed of niet overgedragen worden, of over eigenschappen beschikken die transmissie van hun genetisch materiaal beperken. De wetenschappelijke onderbouwing van deze eigenschappen is in het algemeen goed, hoewel eigenschappen als infectiviteit en transmissie vaak niet kwantitatief zijn bepaald.
- Concentreren en opsluiten; een eenvoudig principe dat beoogt om micro-organismen zoveel mogelijk op te sluiten, het werk met infectieuze micro-organismen zoveel mogelijk te beperken (bijv. door PCR amplificatie te gebruiken in plaats van micro-organismen te kweken), en het aantal werkplekken waar met micro-organismen gewerkt wordt te beperken.
- Blootstelling minimaliseren; een volgende belangrijke stap om de risico's verbonden aan het werken met micro-organismen te beperken, bestaat uit een aantal gedragingen die bekend staan als veilige-microbiologische-technieken (VMT), en die bestaan uit netjes en gedisciplineerd werken, het dragen van beschermende kleding, het voorkomen van de vorming van

aërosolen, etc. In de praktijk wordt hierbij vaak gebruik gemaakt van apparatuur die micro-organismen fysisch inperkt.

- Fysisch inperken; verdere bescherming van de laboratoriummedewerker en de omgeving wordt verkregen door fysische barrières die de ontsnapping van micro-organismen vanuit de werkplek en het laboratorium voorkomen of verminderen. Deze bestaan uit een samenstel van apparatuur en bouwkundige voorzieningen, zoals veiligheidskabinetten, isolatoren, filters, sluizen, etc. Micro-organismen, zowel GMOs als niet-GMOs, worden daartoe in vier gevarenklassen ingedeeld, waarna de werkzaamheden toegekend worden aan een fysisch inperkingniveau, die op hun beurt ook weer in vier klassen zijn ingedeeld. Deze indeling is afhankelijk van de risico assessment ten aanzien van het micro-organisme en de genetische modificatie. Daarnaast kunnen aanvullende maatregelen genomen worden.
- Gevaren minimaliseren; als met bovengenoemde methoden risico's geminimaliseerd zijn, dan kunnen tenslotte de consequenties van expositie aan micro-organismen geminimaliseerd worden als die toch optreedt. Hieronder kunnen maatregelen gerekend worden als het beschikbaar hebben van noodprocedures en het vaccineren van medewerkers.

Toetsingskader om effectiviteit van biosafety maatregelen te toetsen

In de wetenschappelijke literatuur wordt de effectiviteit van biosafety maatregelen slechts fragmentarisch geëvalueerd. Ook is de totale hoeveelheid literatuur waarin maatregelen geëvalueerd wordt gering. Daarom lijkt er ook geen consensus of een bestaande praktijk te zijn ontstaan die aangeeft hoe de effectiviteit van biosafety maatregelen geëvalueerd dient te worden. Wij geven daarom in bijgaande tabel een toetsingskader aan dat behulpzaam kan zijn bij de beoordeling van de effectiviteit van maatregelen. Hierin wordt de effectiviteit van maatregelen op drie niveaus onderzocht, te weten dat van afzonderlijke apparaten en procedures, dat van de totale laboratoriumomgeving, en dat van de laboratoriummedewerkers en de omgeving. De effectiviteit van de inperkingsmaatregelen kan zowel experimenteel getoetst worden, bijvoorbeeld door het experimenteel valideren van apparatuur en het laboratoriumontwerp met modelmicro-organismen of partikels, als onder echte praktijkomstandigheden.

Toetsingskader om de effectiviteit van biosafety maatregelen te evalueren

	Experimentele condities	**Praktijk omstandigheden**
Apparatuur en procedures	*Geven afzonderlijke apparaten en procedures effectief bescherming tegen experimentele blootstelling aan deeltjes of model micro-organismen?*	*Geven afzonderlijke apparaten en procedures effectief bescherming tegen blootstelling aan micro-organismen tijdens het praktisch werk?*
Laboratorium	*Geeft de totale laboratoriumomgeving effectieve inperking van micro-organismen na experimentele blootstelling aan deeltjes of model micro-organismen?*	*Geeft de totale laboratoriumomgeving effectieve inperking van micro-organismen tijdens het praktisch werk?*
Laboratoriummedewerkers en omgeving	*N.v.t.*	*Zijn laboratoriummedewerkers en de omgeving daadwerkelijk beschermd tegen infectie?*

Hoe effectief zijn biosafety maatregelen?

Zoals al aangegeven is er weinig literatuur waarin de effectiviteit van biosafety maatregelen op systematische wijze geëvalueerd wordt. Wij maken gebruik van bovengenoemd toetsingskader om één en ander samen te vatten.

i). Geven afzonderlijke apparaten en procedures effectief bescherming tegen blootstelling aan micro-organismen?

Omdat veel laboratoriuminfecties overgedragen worden via aërosolen, is er in de literatuur met name, en ook nagenoeg uitsluitend, aandacht besteed aan de evaluatie van biologische veiligheidskabinetten. Biologische veiligheidskabinetten kunnen de blootstelling van medewerkers aan micro-organismen beperken, maar hun effectiviteit neemt af door verkeerd gebruik, verkeerde plaatsing, of onvoldoende onderhoud. Er is enige zorg omtrent cel-sorterings apparatuur (cell sorters), omdat deze aërosolen vormen en vaak niet in veiligheidskabinetten of ingeperkte laboratoria zijn geplaatst. Met speciale technische voorzieningen wordt getracht aan deze bezwaren tegemoet te komen.

ii). Geeft de totale laboratoriumomgeving effectieve inperking van micro-organismen?

Omdat apparatuur, bouwkundige voorzieningen en procedures niet geïsoleerd werken, maar elkaar vaak aanvullen of als achterwacht functioneren, lijkt het aangewezen om de effectiviteit van al deze voorzieningen integraal te evalueren. Er zijn echter slechts enkele studies verschenen die rapporteren over contaminatie van de laboratoriumruimte en de effectiviteit van maatregelen om die contaminatie tegen te gaan.

iii). Zijn laboratoriummedewerkers en de omgeving daadwerkelijk beschermd tegen infectie?

Het monitoren van laboratoriuminfecties is één van de belangrijkste methoden om de effectiviteit van inperkingsmaatregelen te evalueren. Bovendien kunnen zij wijzen op te nemen maatregelen om tekortkomingen te verbeteren. De incidentie van laboratoriuminfecties lijkt gering en ook een dalende tendens te vertonen. Hoewel sommige landen beschikken over surveillance-systemen voor laboratoriuminfecties, lijkt er sprake te zijn van sterke onderrapportage. De literatuur bevat voornamelijk case reports en enkele retrospectieve studies. Opvallend is dat de literatuur melding maakt van vele infecties met niet-GMOs (opgetreden gedurende tientallen jaren) en slechts van enkele infecties met GMOs, die dan ook nog beperkt zijn tot infecties met recombinant vacciniavirus. Deze grote discrepantie wijst waarschijnlijk op de grote mate van effectiviteit van biologische inperking die veel GMOs karakteriseren. Juist

vacciniavirus is echter in geringere mate biologisch ingeperkt en nog in zekere mate virulent. Andere factoren die het geringe aantal accidenten met GMOs kunnen verklaren zijn waarschijnlijk de grote bekendheid met de eigenschappen en risico's van bepaalde GMOs, de geringere hoeveelheid werk die uitgevoerd wordt met GMOs, en de striktere praktijk en regelgeving rond het werken met GMOs. Laboratoriuminfecties met niet-GMOs worden vooral veroorzaakt door pathogene micro-organismen die een lage dosis nodig hebben om infectie te bewerkstelligen. Soms is een duidelijke oorzaak voor een laboratoriuminfectie aan te wijzen, zoals het niet volgen van de juiste werkvoorschriften, prikaccidenten of risicovolle handelingen met proefdieren, maar in de meeste gevallen zijn zulke oorzaken niet aan te geven. Mogelijk zijn duidelijke oorzaken dan niet opgemerkt, maar dit zou ook kunnen wijzen op onvoldoende effectiviteit van inperkingsmaatregelen, bijvoorbeeld leidend tot infectie via aërosolen. Ondanks hun beperkingen kunnen retrospectieve studies wijzen op lacunes in de biosafety praktijk. Zo wees een recente studie naar laboratoriuminfecties veroorzaakt door meningokokken duidelijk op het belang van respiratoire bescherming.

Conclusies en aanbevelingen

Maatregelen die gericht zijn op het beheersen van de risico's die verbonden zijn aan het werken met GMOs zijn in de loop van de vorige eeuw ontwikkeld. Ze berusten op en zijn grotendeels identiek aan de maatregelen voor pathogene niet-GMOs. De set van maatregelen is ontwikkeld op basis van ervaringen, gezond verstand en het oordeel van experts. De effectiviteit van biosafety maatregelen is echter nauwelijks en weinig systematisch geëvalueerd. Met uitzondering van biologische inperking is er weinig evidence voor de gegroeide biosafety praktijk. De bijdrage van afzonderlijke maatregelen is daarom meestal onduidelijk. Ook zijn de doelstellingen van inperkingsmaatregelen en voorschriften vaak niet expliciet vermeld. Veel maatregelen zijn in wet- en regelgeving vastgelegd, maar er zijn ook veel details niet geregeld en doelvoorschriften geformuleerd. Hierdoor ligt er nog een grote verantwoordelijkheid bij onderzoekers, de COGEM en vergunningverleners. Zij moeten onder andere rekening houden met gen-gen en gen-omgevings-interacties.

Ook al ligt er niet een heel solide kennisbasis ten grondslag aan de biosafety praktijk, het lijkt niet aangewezen om de huidige praktijk drastisch te wijzigen. Veel maatregelen kunnen effectief zijn en het aantal laboratoriuminfecties lijkt niet erg groot, ook al is er sprake van onderrapportage. Wel bevelen wij aan om bij het vereenvoudigen en moderniseren van richtlijnen zoveel mogelijk uit te gaan van maatregelen met bewezen effectiviteit en de daarvoor benodigde gegevens te

verzamelen. Tevens kunnen de doelstellingen van biosafety maatregelen explicieter geformuleerd worden teneinde naleving en evaluatie te bevorderen. Omdat zowel de risico's als de instrumenten om die te beheersen in grote mate overeenkomen, bevelen wij aan om één enkele set van regelgeving voor GMOs en niet-GMOs te ontwikkelen. Ook bevelen wij aan om de regelgeving van verschillende instanties, zoals EU, WHO, en CDC, verder te harmoniseren.

Laboratoriuminfecties worden veroorzaakt door menselijk of technisch falen in de inperkingsmaatregelen of door het tekortschieten van inperkingsmaatregelen, bijvoorbeeld bij het tegengaan van infectie door aërosolen. Uit de literatuur over laboratoriuminfecties blijkt duidelijk hoe belangrijk het is dat medewerkers goed opgeleid zijn en veilige microbiologische technieken gebruiken. Opvallend is het geringe aantal laboratoriuminfecties veroorzaakt door GMOs. Deze zijn beperkt tot infecties met recombinant vaccinia virus, wat verklaard kan worden door de geringe biologische inperking van dit virus. Wij raden aan om de mogelijkheden van biologische inperking zoveel mogelijk verder uit te buiten. Zo kunnen verder geattenueerde vaccinia virus stammen, zoals MVA, ALVAC en NYVAC, gebruikt worden. Ook voor onderzoekshandelingen met niet-GMOs is het raadzaam om, waar mogelijk, minder virulente stammen te gebruiken.

Het monitoren van laboratoriuminfecties is belangrijk als maat om de integrale effectiviteit van maatregelen te kunnen beoordelen, maar ook om verbeteringen op te kunnen baseren. Wij bevelen daarom aan om deze monitoring te optimaliseren en te bevorderen dat de meldingsgraad omhoog gaat. Het zogenaamde "blame-free" melden, dat de melder vrijwaart van negatieve consequenties, kan hierbij een belangrijk hulpmiddel zijn.

Het verder onderbouwen van de wetenschappelijke basis van biosafety kan de effectiviteit van inperkingsmaatregelen en het naleven ervan bevorderen. Het zal echter veel tijd en moeite kosten om de biosafety praktijk volledig "evidence-based" te maken, voorzover dat al haalbaar en noodzakelijk is. Vanuit de wetenschap bezien liggen er wel grote uitdagingen om de effectiviteit van afzonderlijke maatregelen en hun onderlinge samenhang te onderbouwen. Mathematische modellen, waarin kwantitatieve parameters van infectiviteit en transmissie een cruciale rol spelen, kunnen hierbij behulpzaam zijn.

Samenvatting van de aanbevelingen

- Versterk bij het moderniseren van de regelgeving waar mogelijk en haalbaar de “evidence” van de effectiviteit van inperkingsmaatregelen. Hierdoor kan zowel de effectiviteit van de maatregelen als de naleving bevorderd worden.
- Ontwikkel één enkele set van regelgeving voor GMOs en niet-GMOs.
- Vermeld expliciete doelstellingen van inperkingsmaatregelen en voorschriften.
- Baseer waar mogelijk kennis en maatregelen ten aanzien van bioveiligheid op kwantitatieve parameters van infectiviteit en transmissie-eigenschappen van micro-organismen.
- Bevorder het verder harmoniseren van de regelgeving van verschillende instanties, zoals EU, WHO, en CDC.
- Bij de risico assessment dient rekening gehouden te worden met gen-gen en gen-omgevings-interacties.
- Maak zoveel mogelijk gebruik van de mogelijkheden van biologische inperking, met name bij recombinant vaccinia virus.
- Bewaak en evalueer veiligheidaspecten in laboratoria en de naleving van regels regelmatig.
- Optimaliseer de monitoring van laboratoriuminfecties en implementeer bevindingen naar aanleiding van zulke infecties. Serologisch onderzoek kan het opsporen van laboratoriuminfecties ondersteunen en dient in overeenstemming te zijn met de risico’s. “Blame-free” melden, dat de melder vrijwaart van negatieve consequenties, kan een belangrijk hulpmiddel zijn om de meldingsgraad te verhogen.
- Bevorder de opleiding van labortoriumpersoneel en het naleven van regels.
- Bevorder het verzamelen van gegevens die de wetenschappelijke basis van inperkingsmaatregelen kunnen onderbouwen.
- Mathematische modellen, waarin kwantitatieve parameters van infectiviteit en transmissie een cruciale rol spelen, kunnen behulzaam zijn bij het verder ontwikkelen van onze kennis over inperkingsmaatregelen, bij het signaleren van kennislacunes, en bij het verder ontwikkelen en evalueren van inperkingsmaatregelen.

Summary

Background. Working with pathogenic microorganisms and genetically modified microorganisms (GMOs) requires precautions that guarantee the safety of man and the environment.

Objective. To examine available evidence of the effectiveness of measures aimed at protecting man and environment against the risks of working with GMOs and with non-GMO pathogenic microorganisms.

Methods. Systematic literature review.

Results. Both the nature of risks and measures to handle risks are largely identical for GMOs and non-GMOs. However, in many countries there are different regulations for GMOs and non-GMOs, which may be confusing to workers in the field. A few principles and methods appear to underlie the current biosafety practice: risk assessment, biological containment, concentration and enclosure, exposure minimization, physical containment, and hazard minimization. This results in a set of universal precautions employing a classification of microorganisms in four hazard classes and associated standard biosafety practices. These are a composite of design features, construction, containment equipment, and operational procedures. Much of these practices are based on experience and expert judgment. Effectiveness of biosafety measures may be evaluated at the level of single containment equipment and procedures, the laboratory as a whole, or at the clinical-epidemiological level, and both under experimental conditions to test and validate equipment, and actually during practical work. Data on the containment effectiveness of equipment and laboratories is scarce and fragmented, and mainly limited to technical specifications. Laboratory-acquired infections (LAIs) are therefore important for evaluating effectiveness of biosafety, and they may provide important lessons for optimizing safe conditions. Monitoring of LAIs is usually not done systematically, and suffers from serious underreporting. In some cases of LAIs non-compliance with biosafety rules is obvious. However, in the majority of cases there appears to be no direct cause, suggesting that failures were not noticed, or that containment may have been insufficient resulting in infection through, for example, exposure to aerosols. The number of reported laboratory accidents associated with GMOs is substantially lower than those associated with non-GMOs, possibly because many GMOs display a high level of biological containment. Other factors may be a lower quantity of work involving GMOs, a stricter regulatory framework, a stricter compliance to containment rules, and the absence of unknown pathogens in GMO laboratories.

Conclusions. While together the measures directed at assuring biosafety of pathogenic microorganisms and GMOs appear largely effective, it is unknown to

what extent specific measures contribute to the overall level of biosafety. To optimize the effectiveness of biosafety and to stimulate compliance with safety rules, we recommend to strengthen the evidence-base of the biosafety practice where possible and feasible by defining criteria to evaluate effectiveness, by further acquiring data on the effectiveness of containment measures, and by optimizing monitoring of LAIs. Knowledge and measures of biosafety should be directed on, preferably quantitative, parameters of infectivity and transmission. We recommend to develop a single set of regulations for GMOs and non-GMOs. Whenever possible, we consider it important to further optimize the possibilities of employing genetic modification to enhance biological containment of GMOs, in particular with respect to recombinant vaccinia virus. Routine evaluation and monitoring of biosafety aspects are advisable to enhance the overall safety awareness. Education of laboratory personnel and compliance to the rules remain important. Scientific challenges are to estimate the contribution of single measures to biosafety and their mutual relationships. For that purpose mathematical models may be supportive. These models may estimate the contribution of several measures to safety, point to gaps in our knowledge, and support the development and evaluation of biosafety measures.

1. Introduction

Working with pathogenic microorganisms and genetically modified microorganisms (GMOs) requires precautions that guarantee the safety of man and the environment, including laboratory personnel, patients treated with GMOs, and other persons that could be exposed to these microorganisms. During the past decades responsible authorities and researchers have therefore developed regulations and guidelines that in some detail describe containment measures and working instructions (Fig. 1). For GMOs such regulations and guidelines appear largely derived from those developed for working with the natural, genetically unmodified pathogenic microorganisms from which these GMOs have been derived.

Despite containment measures and guidelines, laboratory infections, usually involving non-GMOs, occur more or less common, suggesting that biosafety rules are not always effective or not complied with. The guidelines and instructions for working with GMOs appear largely effective as there have been no major accidents with GMOs or with their unintended release. Nonetheless, despite such regulations and the lack of major accidents with GMOs, there appears to be continuing concern about the health and safety of individuals and the environment exposed to potentially hazardous GMOs (Keatly 2000). It has also been noted that the laws and regulations governing the biotechnology world are outdated, are not comprehensive, and span too many agencies (Keatly 2000). Indeed, while the nature of risks and measures to handle these risks are largely identical for GMOs and non-GMOs, there are in many countries different regulations for GMOs and non-GMOs. This may be because the latter were presumed to carry greater risks for causing ecological disturbances upon unintended release. For example, in the Netherlands the Ministry of the Environment oversees working with GMO, while the Ministry of Social Affairs oversees working with human pathogens. The different regulations and overseeing authorities may be confusing to workers in the field. Moreover, it is unknown to what extent specific factors contribute to a safe biosafety practice. Thus, it is often unclear if and to what extent measures aimed at providing biosafety are based on documented evidence of their effectiveness.

A central question in this study is whether containment measures are effective and evidence-based. One may argue that the evidence for the effectiveness of containment measures is at best indirect, i.e based on the lack of many overt laboratory-acquired infections (LAIs). In addition, we could question whether the criteria to judge effectiveness are sufficiently developed. Indeed objectives of containment measures are often not explicitly defined, and without (quantifiable) objectives evaluation of effectiveness is difficult. Furthermore, in finding evidence for

the effectiveness of biosafety measures, it is important to judge the quality of the evidence. For comparison, in evidence-based medicine systematic reviews, hypothesis-driven controlled laboratory experiments, and prospective studies provide a higher quality of evidence in comparison with case reports and expert opinion.

In this review we will give a brief historical overview of the development of the current biosafety practice, we will try to identify which principles and methods appear to underlie it, and we will describe this current biosafety practice. Subsequently we will present an approach for evaluating the effectiveness of biosafety measures to contain pathogenic microorganisms, and finally we will summarize experimental and observational data on the effectiveness of containment measures. Our primary goal is to evaluate the evidence-based containment measures for GMOs. However, because such measures are largely based on containment measures for non-GMOs and because more data, although scarce, are available for non-GMOs, we also examine evidence-based measures to contain non-GMO pathogens. These data may be extrapolated to GMOs. We therewith hope to contribute to a conceptual framework that helps in further developing an evidence-based biosafety practice.

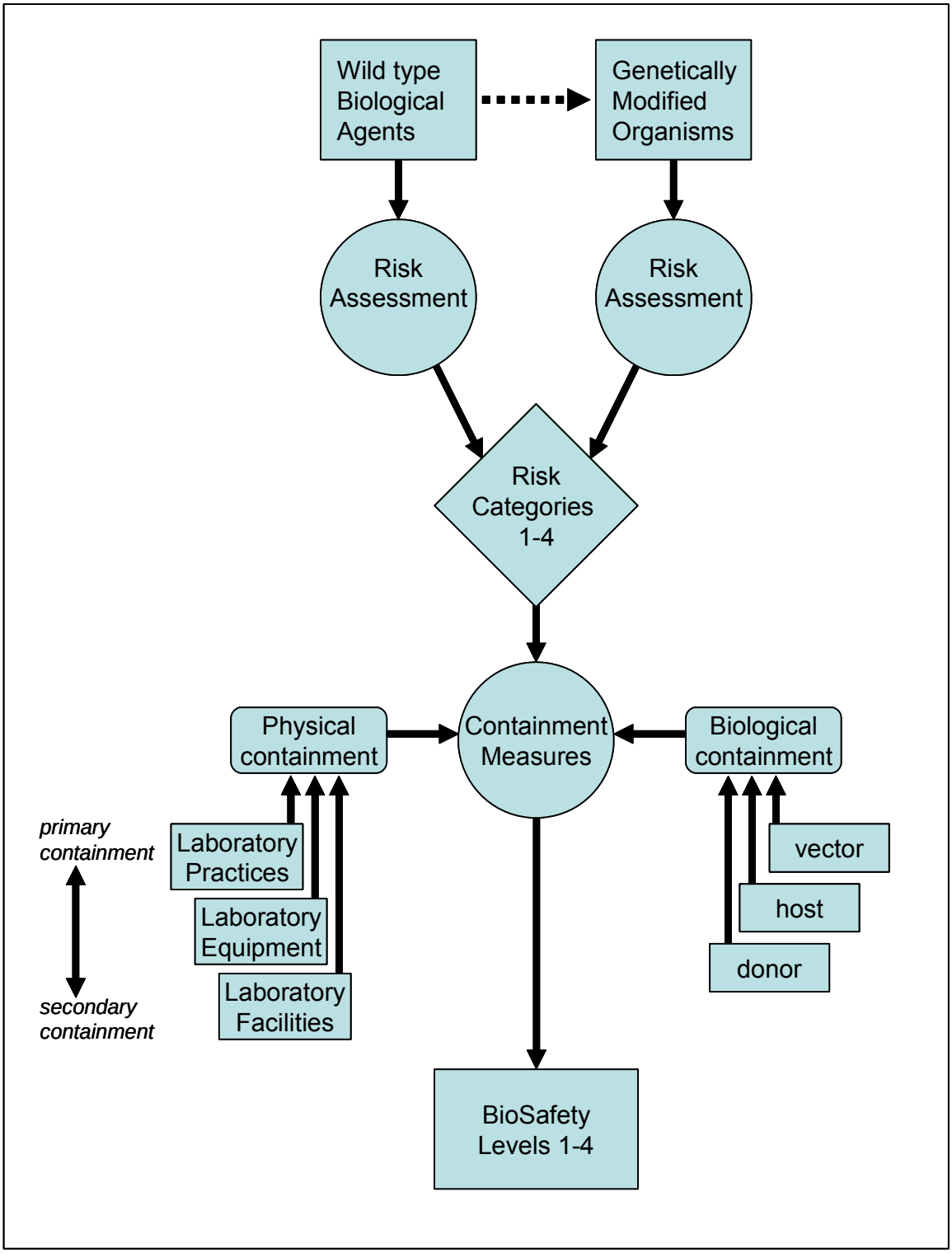

Fig. 1. Context of biosafety measures.

Based on a risk assessment wild-type biological agents and GMOs are assigned to one of 4 risk categories. The work is subsequently performed under conditions that reflect increasing containment demands, i.e. biosafety levels 1-4. Risks are contained by a set of measures employing biological and physical barriers and laboratory practices.

2. Development of containment measures: brief historic overview

While Robert Koch had already developed some kind of biosafety cabinet, Dr. Wedum of the US Biological Research Laboratories at Fort Detrick can be regarded as one of the pioneers in developing biosafety measures after the Second World War. He evaluated risks of handling hazardous biological agents and developed practices, equipment, and facility safeguards for their control (Wedum 1953, Wedum et al 1956, Philips and Runkle 1967). Following his initial work, it is now regarded conventional wisdom that enclosure and ventilation of the contaminated work area are important factors in eliminating LAIs. Besides safe microbiological techniques, primary barriers (safety equipment and personal protective equipment) and secondary barriers (facility safeguards) are now regarded as vital elements of containment measures.

It was early recognized that examining LAIs could be informative on the risks involved with laboratory work. Several comprehensive reviews of LAIs have therefore been compiled (Pike 1979, Sewell 1995). These early examinations recognized that the primary route of transmission of many of the causative agents was by aerosol, and led to the development of laminar-flow biological safety cabinets (BSCs). Legislation and guidelines that were introduced during the years have probably reduced, but not eliminated the risk of occupational risk of exposure to infectious agents.

With the growing ability to manipulate DNA in the mid 1970s, there was also growing concern about the potential hazards associated with recombinant DNA research and technology (Berg et al 1975). Researchers were able to combine genetic material from different sources thereby creating GMOs that never existed in nature before. These GMOs could display the intended properties, but could also have unpredictable and undesirable features. Besides major advances in molecular technologies that have brought breakthroughs in medicine, genetics, agriculture and biology, there is still continuing debate (Keatly 2000, Nelson 2001) about the health and safety of laboratory workers and animals, as well as the environment, exposed to potentially hazardous GMOs. At the Asilomar Conference in 1975, general principles for dealing with potential biohazards related to GMOs were drafted. It was suggested that containment should be an essential consideration in the experimental design and that the effectiveness of the containment should match the estimated risk. Adjustment of the level of precaution to the level of risk would prevent infection without unduly impeding operations. It was suggested that containment of potentially hazardous GMOs could be achieved in several ways, i.e. by using biological barriers,

as well as by physical containment (safety equipment and facility safeguards) complemented by safe microbiological practices.

The first edition of the National Institutes of Health (NIH) guidelines for research involving DNA molecules appeared in 1976. Now 3 decades later there are a number of authoritative international guidelines, instructions and recommendations for the safe handling and manipulation of hazardous biological agents, including GMOs. In 1984, the US NIH and Centers of Disease Control (CDC) produced the first edition of a guidebook, called Biosafety in Microbiological and Biomedical Laboratories (BMBL) that is now considered as a major reference text. The NIH/CDC and the WHO manuals, are based on historical accounts of incidents with infectious microorganisms and extensive experience of experts working in this field and have been developed and improved over the last 30 years (Anon 2004, Wilson and Chosewood 2007). Legislature has been implemented, for example in EU and national regulations (VROM 2004). In Europe national authorities have based their regulations on Directives from the EU, such as the Directive on the protection of workers from risks related to exposure to biological agents at work, and the Directive on the contained use of genetically modified microorganisms. A summary of important guidelines and manuals is given in Table 1.

Table 1. Summary of guidelines and directives on recombinant DNA research

Institution		Issued in	
EEC/EC	Directive on the protection of workers from risks related to exposure to biological agents at work	1990	90/679/EEC, 89/391/EEC, 2000/54/EC
	Directive on the contained use of genetically modified microorganisms	1990	90/219/EEC, 98/81/EC, 2000/608/EC
AS/NZS	Safety in laboratories (Part 3: Microbiological aspects and containment facilities)	2002	2243.3
PHAC	Laboratory Biosafety Guidelines	2004 (3rd ed.)	www.phac-aspc.gc.ca/publicat/lbg-ldmbl-04/index.html
WHO	Laboratory Biosafety Manual	2004 (3rd ed.)	www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en
VROM	Intergrale versie van de Regeling genetisch gemodificeerde organismen en Besluit genetisch gemodificeerde organismen	2004	www.vrom.nl/docs/milieu/regelingGGO_inclusiefbijlagen_1okt2003.pdf

3. Principles and methods of biosafety

In judging the effectiveness of measures intended to ensure biosafety, it may be helpful to know which goals, principles and methods of biosafety measures have been employed, and to evaluate the scientific basis of their effectiveness. Because the nature of risks are largely identical for GMOs and non-GMOs, containment measures to handle these risks are largely identical for both. Altogether biosafety measures have evolved in the past step by step, and usually based on expert knowledge and experience, but without a unifying set of guiding principles. Explicit guiding principles are therefore usually lacking in most legal regulations and scientific papers. We here attempt to draft a hierarchy of such guiding principles and methods that may be applicable to both GMOs and non-GMOs:

1. Risk assessment; this is the first and central step, and includes hazard recognition and identification, understanding of exposure potentials, frequency of occurrence, evaluating work tasks and equipment, and assigning protective measures to the specific tasks involved.
2. Biological containment; wherever possible risks for the workers and the environment may be minimized by reducing exposure potentials and their consequences by using attenuated microorganisms (GMO or non-GMO) that have reduced replicative capacity, infectivity, transmissibility, and virulence.
3. Concentration and enclosure; a simple and often overlooked, but obviously one of the most important ways to provide biosafety is to “lock up” microorganisms as much as possible, to limit the microbiological work load, and to concentrate it in as few as possible work sites. For example, if work associated with a harmful microorganism can be restricted to one biosafety cabinet, it will carry less risk compared to the situation when this work is done in two biosafey cabinets. Detection of microorganisms by nucleic acid multiplication carries less risk than detection by culture.
4. Exposure minimization; when enclosure has to be disturbed, exposure can be minimized by a set of behaviors collectively known as safe microbiological techniques, including an orderly and disciplined work fashion, wearing gloves, wearing a mask and eye covering, prevention of aerosol and droplet generation, no mouth pipetting, prevention of skin disruptions, etc. These measures in particular provide operator protection (Sargent and Gallo 2003). In practice they often make use of special equipment providing physical containment. Laboratory workers should be trained in these aspects.
5. Physical containment; further protection of the operator and the environment is provided by physical barriers that prevent or minimize escape of

microorganisms from the working place and laboratory. These include a range of design requirements such as doors and locks, and physical safety equipment such as BSCs, isolators, air filtration, waste water management systems, etc. In addition to protecting the laboratory workers, these measures provide environmental protection. Obviously such physical containment is combined and complemented by the previous methods. Physical barriers have been designated as primary and secondary barriers. Primary containment measures minimize occupational exposure of laboratory workers and therewith limit transmission of microorganisms from these workers to others. The secondary barriers provide supplementary microbiological containment, serving mainly to prevent the escape of infectious agents when a failure occurs in the primary barriers.

6. Hazard minimization; this includes a set of activities to reduce the consequences of exposure should it occur, and may include the availability of emergency procedures, a contingency plan, health and medical surveillance, but also vaccination to reduce the consequences of inadvertent exposure (Anon, 2004).

In the following sections we further elaborate on some of these principles and methods, and the way in which they have been incorporated in legislature and regulations.

4. Biosafety measures

4.1. Risk assessment

From a thorough risk assessment procedure, considering all potentially harmful effects for man and the environment, follows the risk classification of microorganisms and subsequent containment measures that should be taken to manage these risks. Both the nature and scale of activities need to be considered to estimate the possibility of exposure to humans and the environment, and its consequences. Examples of risk assessment procedures can be found in http://www.hse.gov.uk/biosafety/gmo/acgm/ecrisk.htm and EU Directive 90/219/EEC. As indicated below, there are 4 risk categories for hazardous biological agents and 4 containment levels. Work with non-GMO microorganisms is usually done at the corresponding containment level. Evidently a risk assessment for working with non characterized pathogens in a clinical laboratory may involve uncertainties. Through manipulation GMOs may acquire unexpected and/or not well-characterized or understood pathogenic properties, necessitating a higher containment level than for work with the natural microorganism from which the GMO has been derived, or additional measures. See for example VROM 2004. Therefore the nature of recombinant DNA sequences, vectors and recipient organisms, need to be carefully evaluated, as well as any potential biohazard associated with particular experimental settings. It is particularly important to address whether or not genetic modification affects cell tropism, host range, virulence, or the susceptibility to antibiotics or other effective treatments. Some considerations regarding the risk assessment and categorization of GMO activities are given in Table 2.

Table 2. Summary of considerations in the risk assessment for GMOs

Recipient microorganisms	virulence
	transmissibility
	host range
	susceptibility to antivirals or antibiotics
	availability of prophylaxis, control and treatment
Vectors	replicative capacity
	integration into host genome
Insert or donor sequences	toxicity
	biological properties
	replicative capacity
	properties known/unknown
	gene-gene end gene-environment interactions
Activities	scale
	animal experiments
	transport
Host factors	immunodeficiencies
Population factors	(vaccine-derived) immunity

4.2. Biological containment

A subsequent step in the selection of measures to ensure biosafety is to minimize biological hazards associated with the work by employing host microorganisms with a reduced host range, strains with natural or genetically modified characteristics that diminish their invading capacity or virulence, self-inactivating vectors, etc. Thus employing biological containment is not restricted to work with GMOs. Natural pathogenic microorganisms may be replaced by less pathogenic microorganisms, for example replacing *Mycobacterium tuberculosis* with non-virulent mycobacteria, such as *Mycobacterium smegmatis* (Danelsishivi et al 2006). Approaches of acquiring biological containment by genetic modification are given in Table 3.

Table 3. Principles and methods of establishing biological containment

Principle	*Method*	*Example*
Attenuation	natural or genetically modified deletion of virulence genes	modified vaccinia virus Ankara, herpesvirus vectors, *E. coli* K12, *Salmonella aro* mutants, *Vibrio* ctx mutants, *Lactococcus lactis thyA* mutant
Host range restriction	natural host-restricted viruses	canarypox, fowlpox, baculovirus
Host range alteration	ecotropic packaging cell lines, pseudotyping	retroviral vectors
Replication-defective vectors	deletion and providing essential gene products *in trans*;	herpesvirus vectors, alphavirus vectors, retroviral vectors, adenovirus vectors, adeno-associated viral vectors, respiratory syncytial virus, lentivirus vectors *Salmonella aro* mutants, *E. coli* K12
Prevention of gene transfer	expression of suicide functions	*E. Coli relF*

4.2.1. *Viruses*

Examples of biological containment include the use of highly modified vaccinia virus Ankara (MVA), which has a significantly reduced pathogenicity, and the use of avian poxviruses, including canarypox and fowlpox, that have a restricted host range and do not replicate in mammals (Moss 1996, Paoletti et al 1996). These viruses have a better safety profile than the classical vaccinia virus.

Biological containment for retroviral vectors has been obtained by providing gene products that are required for the production of progeny viruses, i.e. *gag*, *pol*, and *env,* in *trans* by packaging cell lines that stably express these *trans*-acting functions. Formation of replication-competent HIV is excluded when the *env* gene is missing from the packaging plasmid. Furthermore, the range of host species that can be transduced with such vectors can be manipulated by using particular *env* genes or by pseudotyping with envelope proteins of other viruses (Debyser 2003, Farson et al 1999, Romano et al 1999). Various envelope proteins are not only associated with a varying host range, but also with varying stability and intrinsic toxicity. A concern has been that recombination events may give rise to replication-competent lentiviruses, but further modifications have been made to reduce this opportunity. These include the use of different transcriptional units, further attenuation by deleting non-essential genes, and self-inactivating vectors (Bukovsky et al 1999, Debyser 2003). Self-inactivating vectors contain an inactive long terminal repeat (LTR), resulting in inactive promoter activity and preventing potential transcriptional activation of (onco)genes downstream of the integration site. These vectors cannot be rescued by wild-type HIV. No replication-competent lentiviruses have been detected when using such systems (Debyser 2003, Escarpe et al 2003). Altogether the risk associated with the newly developed lentivectors is therefore minimal, while they have distinct advantages such as stable integration in non-dividing and dividing cells, long-term expression of the transgene, and absence of an immune response. However, although viral infection using state-of-the art lentivectors is very unlikely, their high transduction efficiency may increase the risk of accidental exposure of the lab worker leading to a positive anti-p24 HIV antibody response and accidental transduction of potentially hazardous genes. Contact transmission thus should still be avoided.

Similarly, replication-deficient adenoviruses have been developed using a viral DNA vector and a packaging cell line that has been stably transfected with the E1A region of the adenoviral genome. Vectors prepared from the cell line lack the E1A region and remain replication-defective (Kost et al 2000).

Safe replication-deficient herpesvirus vectors have been developed by deleting the viral glycoprotein gD. Envelope glycoprotein gD is essential for virus entry, but is not

required for subsequent steps in the viral replication cycle. Phenotypically-complemented gD null mutants can infect cells and can spread, both *in vitro* and *in vivo*, by direct cell-to-cell transmission. However, progeny virions released by the infected cells are non-infectious because they lack gD (Peeters et al 1994). Thus an accidental infection remains restricted to a single round of replication. The same principle has been used for other viruses, for example respiratory syncytial virus. Other ways to generate safe herpesvirus vectors are deletion of genes that are essential for virulence *in vivo*, but that are non essential *in vitro*, or to delete immediate early genes that activate early and late gene expression and subsequent propagation of the crippled virus in complementing cell lines (Kimman et al 1995, Laquerre et al 1999). Replication-incompetent herpesvirus vectors have also been generated by the use of cosmid DNAs to provide the necessary viral gene products for propagation of defective viruses or amplicons. These approaches reduce the opportunities for generation of replication-competent viruses through recombination.

Safe vectors have been developed from the non-pathogenic adeno-associated virus. The vector lacks all viral genes, and requires coinfection with a helper adenovirus or a helper-free packaging system (Collaco et al 1999, Samulski et al 1999).

A high level of biological containment is also acquired by using the *Autographa californica* nuclear polyhedrosis virus (AcMNPV), a member of the baculovirus family. These viruses normally replicate in insect cells, but not in mammalian cells. Furthermore, by deleting the nonessential polyhedron gene, the virus becomes noninfectious for its natural host (O'Reilly et al 1994).

4.2.2. *Bacteria and protozoa*

The classical example of a biologically contained bacterium is *E. Coli* K12. *E. coli* K-12 is a debilitated strain that does not normally colonize the human intestine. The strain survives poorly in the environment and has a history of safe commercial use. *E. coli* K-12 is considered an enfeebled organism as a result of being maintained in the laboratory environment for over 70 years (Williams-Smith, 1978). *E. coli* K-12 is defective in at least three cell wall characteristics. The outer membrane has a defective lipopolysaccharide core which affects the attachment of the O-antigen polysaccharide side chains (Curtiss, 1978). Second, it does not have the type of glycocalyx required for attachment to the mucosal surface of the human colon (Edberg, 1991) as a result of the altered O-antigen properties noted above. Finally, K-12 strains do not appear to express capsular (K) antigens, which are heat-labile polysaccharides important for colonization and virulence. K-12, thus, is not able to recognize and adhere to the mucosal surface of colonic cells (Curtiss, 1978). The

normal flora in residence in the colon thus can easily exclude K-12. Furthermore K-12 lacks other virulence factors (Curtiss, 1978, Gorbach 1978, Edberg, 1991).

Several approaches have been used to develop safe bacterial vectors for use as vaccine or gene or protein delivery, in particular strains of *Salmonella, Shigella, Listeria, Mycobacterium, Vibrio,* and lactic acid bacteria (Kochi et al 2003, Steidler 2004). When developing vector vaccines, the challenge is to develop strains that are well tolerated by the recipient host, no longer persist in the environment, yet still induce protective immune responses. This is, however, not always achieved (Dilts et al 2000, Tacket et al 1999). Elucidation of biosynthetic pathways has led to the development of *Salmonella* vectors that were attenuated by disruption of genes encoding metabolic functions or genes located in a pathogenicity island (*Salmonella* Pathogenicity Island-2 that encodes a type III secretion system). The *Salmonella* Pathogenicity Island-2 is required for survival and growth within macrophages (Khan et al 2003). For example, attenuating mutations in *Salmonella* strains included *msbB, galE*, *via, rpoS*+, *aroCD, htrA, cya, crp, cdt, asd, phoPQ, purB, sifA, ssaV* (Kahn et al 2003, Low et al 1999, Michael et al 2004, DiPetrillo et al 1999). The best characterized-live attenuated *Salmonellae* have mutations in the prechorismate pathway. These are the so-called *aro* mutants that are defective in the production of chorismate, which is essential in the synthesis of aromatic compounds (Michael et al 2004). Evidently strains carrying different mutations differ in properties such as invasiveness and survival. To reduce the possibility of reversion to virulence, strains have been produced carrying at least two attenuating distantly located mutations (Khan et al 2003, Tacket et al 1997). *S. typhimurium* VNP20009 was developed to deliver potential therapeutic proteins to tumour sites. It was created by chromosomal deletion of two genes, *purI* (purine biosynthesis) and *msbB* (LPS biosynthesis) and was attenuated at least 10,000 fold in mice compared with the parental wild-type strain (Low et al 1999).

A promising *Listeria* vector vaccine is a *L. monocytogenes* auxotrophic mutant with deletions in alanine racemase (*dal*) and D-amino acid aminotransferase (*dat*), two genes required for the biosynthesis of bacterial cell walls. The strain was highly attenuated in mice (Thomson et al 1998). The strain requires D-alanine to grow and survive. Another *L. monocytogenes* candidate vaccine strain (LH1169) contained deletions in *actA* and *plcB*, genes that are necessary for cell-to-cell spread and escape from secondary vacuoles, respectively (Angelakopoulos et al 2002). Deleting lecithinase activity in *L. monocytogenes* also results in inhibited cell-to-cell spreading (Dietrich et al 1998).

The virulence of *Vibrio cholerae* is mainly due to the expression of cholera toxin (CT). Hence, strategies for attenuating *V. cholerae* for use as expression vector for heterologous antigens have been to engineer mutants in which the CT gene (ctx) or the CT genetic element have been partially or completely deleted (Kochi et al 2003).
Stable mutant strains *of Bacillus licheniformis*, an industrially exploited species, were obtained by introducing defined deletions in *recA* and/or an essential sporulation gene (*spoIV*). These strains are totally asporogenous and severely affected in DNA repair, and therefore UV-hypersensitive. In liquid media these strains grow equally well when compared to the wild type. Hence, such genes appear to be suitable disruption targets for achieving biological containment (Nahrstedt et al 2005).
A genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin-10 (IL-10) employed a biological containment system by replacing the thymidylate synthase gene *thyA* with a synthetic human *IL-10* gene. When deprived of thymidine or thymine its viability dropped considerably preventing its survival in the environment. Transgene escape through acquisition of an intact *thyA* gene is very unlikely and would recombine the transgene out of the genome. The system was validated *in vivo* in pigs (Steidler et al 2003)) and used in a gene therapy study (Braat et al 2006).
Further improvement in enhancing the safety profile of bacterial vectors for gene transfer can be achieved by removing undesirable properties of plasmids, such as a prokaryote origin of replication and antibiotic resistance makers. These elements could lead to dissemination of prokaryotic replicative recombinant DNA. Darquet et al (1997) therefore developed so-called minicircles, supercoiled DNA molecules that lack such elements and only contain an expression cassette carrying the gene of interest. Furthermore, efficient suicide functions have been developed to ensure biological containment of bacteria (Schweder et al 1992, Knudsen et al 1995). Such systems achieve their goals when the GMOs self-destruct by expression of killing genes after fulfilling their jobs. Suicide systems are based on “lethal genes” that are triggered by preprogrammed conditions. Such systems however appear to differ in efficiency of the suicide function, and the less efficient ones may lead to selection of mutants that have lost their suicide function. However no system can provide complete efficiency. One efficient system was based on the lethal *E. coli relF* gene, which prevents the transfer of plasmids to wild-type bacteria (Knudsen et al 1995).
To enhance safety of genetically modified yeast (*Saccharomyces cerevisiae*) genes encoding bacterial toxins have been used for containment control. Expression of the *E. coli relE* toxin gene was highly toxic to yeast cells, and this could be counteracted by expression of the *relB* gene (Kristoffersen et al 2000).

In conclusion, we regard that the mechanisms underlying biological containment are usually well examined and understood. Evidence for their effectiveness is usually available, although only seldom in quantitative terms of infectivtivity and transmission. It is also important to note that not all GMOs are per definition biologically contained. They only have reduced transmissibility or virulence upon disruption of virulence factors. For example, very virulent recombinant influenza and herpes viruses have been regenerated (Grimm et al 2007, de Wind et al 1994).

4.3. Physical containment

4.3.1. *Categorization of microorganisms (non-GMOs)*

Central to biosafety programs is the concept of universal precautions (Buesching et al 1989). For that purpose microorganisms are categorized into four risk categories as a result of a risk analysis. Subsequent risk containment is not focused on specific infectious agents, but on standard practices for handling infectious material that will prevent the transmission of all pathogens of that risk category. It is important to note that principles, guidelines and recommendations are basically the same for natural pathogens and GMOs. However, as would be logical, this conclusion did not lead to a single set of guidelines and recommendations for GMOs and non-GMOs, and thus to some redundancy in guidelines and regulations.

The four categories for biological agents are based on their relative risk for laboratory workers and the community. In general the following factors are considered to classify biological agents: 1) virulence of the biological agent or the severity of disease (in humans) (Casadevall and Pirofski 1999), 2) mode of transmission – spread in the community and host range, 3) availability of effective preventive measures (e.g. vaccines), and 4) availability of effective treatment (e.g. antibiotics, anti-viral drugs). The hazard of the infectious (non-GMO) agent increases from risk group 1, consisting of microorganisms not associated with disease, to risk group 4. Risk group 4 microorganisms can cause serious disease, can be readily transmitted, and effective treatments are usually not available (Wilson and Chosewood 2007, Anon 2004, Schellekens 2001). However, there are differences in the exact definitions as used by certain countries and/or organizations (such as NIH/CDC, WHO, and EU) (table 4), which result in differences in the exact listings of biological agents per risk category (http://www.absa.org/XriskgroupsX/index.html, Flemming 2000). The main difference between the NIH/CDC classification and the WHO classification is that the latter includes hazards to animals and the environment. Another difference is that for risk group 3 the NIH/CDC states "… therapeutic interventions **may be available**", whereas the WHO and EU state that "… effective

treatment and preventive measures **are available**". There also differences in the description of transmission properties between the different classifications. In assigning an agent to a risk group, one must take into account that there are in all groups of microorganisms naturally occurring strains that vary in virulence, and that may thus need a higher or lower level of containment (Flemming 2000). In general regulators deal with this concept by taking the highest level of virulence into account.

There has been debate about the classification of particular microorganisms, in particular the flaviviridae, variola virus, avian influenza A/H5N1, and extremely drug-resistant (XDR) *M. tuberculosis* strains, esp. whether they should be categorized as category 3 or 4 biological agent (CDC 2001, Corbett et al 2003, Fenner et al 1988, Kobasa et al 2004, Nalca et al 2005, Tumpey et al 2005). Because vaccination was stopped in the 1970s, variola viruses are now classified as category-4 biological agents. Although initially avian influenza A/H5N1 strains were classified as risk category-4 biological agents, susceptibility to anti-viral drugs and the availability of effective vaccines may downgrade them to category-3 for further studies. Extremely drug-resistant (XDR) *M. tuberculosis* strains (Corbett et al 2003) should be regarded as risk category-4 biological agent. Of note, to date no other microorganisms other than viruses have been classified under category-4.

Table 4. Classifications of infectious (non-GMO) agents into risk groups by NIH/CDC and WHO

Risk group classification	NIH/CDC	WHO	EU 90/679, VROM
Risk group 1	Agents that are not associated with disease in healthy adult humans	A microorganism that is unlikely to cause human or animal disease	A microorganism that is unlikely to cause human disease
Risk group 2	Agents that are associated with humans disease which is rarely serious and for which preventive or therapeutic interventions are often available.	A pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection but effective treatment and preventive measures are available and the risk of spread of infection is limited	A pathogen that can cause human disease but is unlikely to be a serious hazard to laboratory workers. The risk of spread of infection is limited, and effective treatment and preventive measures are available.
Risk group 3	Agents that are associated with serious or lethal human disease for which preventive of therapeutic interventions may be available.	A pathogen that causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available.	A pathogen that causes serious human disease and pose a serious hazard to laboratory workers. It is likely to spread from one infected individual to another. Effective treatment and preventive measures are available.
Risk group 4	Agents that are likely to cause serious of lethal human disease for which preventive or therapeutic interventions are not usually available.	A pathogen that usually causes serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.	A pathogen that usually causes serious human disease and that can be readily transmitted in the population. Effective treatment and preventive measures are not usually available.

Wilson and Chosewood, 2007) and WHO (Anon, 2004).

4.3.2. *Categorization of GMOs; definition of harmful gene products and microorganisms*

As for non-GMOs, GMOs are assigned to specific risk categories based on the risk assessement (see for example VROM 2004). For GMOs biosafety containment levels are assigned depending on the risk category of the donor organism, unless the modification may result in a higher or unknown risk. Therefore the nature and function of insert sequences, and the properties of acceptor microorganisms are considered. It is remarkable that definitions of properties of GMOs are sometimes quite strict, for example the toxicity of vertebrate toxins, which are expressed in LD50/bodyweight, while other properties of harmful gene products are not exactly defined. In the regulations both the nature and level of virulence and transmissibility of GMOs are not well defined, at least not in quantitative terms (such as the basic reproduction ratio or R_0 [Breban et al 2007]). Sometimes host range is taken into account (for example baculovirus, ecotropic murine retroviruses, and papilloma viruses when they are used in non-permissive host/vector systems). Properties may be unknown, such as the capacity of microbial DNA to integrate into the host genome (as for example HIV) and the availability of vector organisms in the environment and therefore the possibility of persistence in the environment. A point for consideration is therefore the possibility to monitor replication and survival of the GMO. In case of scientific uncertainty the precaution principle is leading, resulting in higher categorization of the GMO or additional measures at a case-by-case base. Although the possibility of microbial transmission is taken into account, the possibility that transmission is reduced by herd immunity, either vaccine-derived or not, is not mentioned explicitly by legislators. The assessment should include whether properties of inserted sequences can be expressed in the background of the host organism. Thus gene-gene and gene-environment interactions should always be considered. As example, interleukin 4 (IL-4) is not normally considered a harmful gene product, but when expressed by a murine ectromeliavirus, it drastically enhanced virulence of this virus by inducing changes in cytotoxic T cell function (Jackson et al 2001). Importantly the regulations, being not completely detailed, largely function as a framework that must be elaborated by researchers and laboratory directors both in their risk assessment, the risk categorization of GMOs, as well as in the implementation of procedures and regulations in their laboratory (Li et al 2005). While the considerations in the risk assessment and subsequent risk categorization may show differences for GMOs and non-GMOs, many of the subsequent containment procedures are similar.

4.3.3. *Laboratory design, primary and secondary containment*

Briggs Phillips and Runkle (1967) describe some principles underlying laboratory design. They introduce two concepts in designing laboratories. The first is the concept of primary and secondary barriers as described above, and the second concept provides the designers a logical division of major functional zones within a laboratory building. (**Note:** although elements of primary and secondary barriers are clearly recognizable, they are not mentioned as such in the European and Dutch regulations.) Briggs Phillips and Runkle (1967) identify five functional zones in the facility (clean and transition, research area, animal holding, laboratory support, engineering support). Primary containment measures minimize occupational exposure of laboratory workers. In addition to strict adherence to good microbiological practice, the primary containment barrier include physical separation of the biohazardous agent from the laboratory worker using closed vessels, personal protective equipment (e.g. gloves, full-body suits) and additional equipment (e.g. BSCs, enclosed centrifuge containers, pipetting aids). The secondary barriers provide supplementary containment, serving mainly to protect other facility employees and to prevent the escape of infectious agents from the laboratory if and when a failure occurs in the primary barriers. These provide a separation between potentially contaminated areas in the building and the outside community. These measures may comprise special procedures (e.g. validated decontamination methods, training of personnel, strictly controlled access zones, interlocked doors, etc) and special engineering and facility design features (e.g decontamination equipment, showers, autoclaves, dedicated air handling system with filters, etc).

Secondary containment is very strict in high-containment laboratories. The high-containment laboratories (BSL 3-4) are airtight, have airlocks and a unidirectional airflow so that potentially contaminated air is kept inside. Thus BSL-3 and 4 laboratories need to be negatively pressured resulting in an air flow from adjacent areas into the laboratory and a filtered exhaust airflow outside the building without recirculation. Ten to 12 air changes per hour have been recommended, which removes approximately 99 % of airborne particles in 23 minutes (Mortland and Mortland 2003). Release of air into the environment is only possible through HEPA (High-Efficiency Particulate Air) filters (rated 99.99% efficient with particles 0.3 microns and larger in diameter) or ULPA (Ultra-Low Penetration Air) filters (rated 99.999% efficient with particles 0.12 microns in diameter). Filters are used for biosafety cabinets, autoclaves, incinerators, chemical decontamination showers, etc. In addition, HEPA filters are used to provide clean air to laboratory workers in full-body suits.

4.3.4. *Categorization of biosafety containment levels*

Biosafety containment levels have been categorized ranging from 1-4. As indicated above, the containment levels are assigned depending on the risk group of the microorganism (GMO or non-GMO), and the scale and nature of activities. Importantly, the biosafety level designations are based on a composite of the design features, construction, containment facilities, equipment, and operational procedures required for working with agents from the various risk groups (WHO 2004). It is important to stress that, although handling microorganisms of a certain risk group usually requires working at the accompanying biosafety level, a risk assessment should be made to take other specific factors into consideration. For example, particular experiments may generate high concentration aerosols, requiring a higher degree of safety. Thus professional evaluation, based on personal responsibility, should always guide the biosafety level for the specific work (WHO 2004). Sewell (1995) formulates some broad recommendations. BSL-1 is recommended for teaching activities with agents that are not associated with disease. BSL-2 practices are used in diagnostic laboratories that manipulate agents that are not transmitted via aerosols (e.g., HBV, HIV, enteric pathogens, and staphylococci). BSL-3 is recommended when working with agents that are highly infectious and are transmitted via aerosols (e.g., *M. tuberculosis*, *Brucella spp*., and *Coccidioides immitis*) and for large-scale work with BSL-2 agents. BSL-4 practices are required when working with unusual agents that cause life-threatening infections for which no treatment is available.

Depending on country and/or regulation authority there are differences between the exact requirements for each of the 4 containment levels, not to mention the sometimes confusing differences in nomenclature for the (high) containment laboratories. Nulens and Voss (2002) review the basic practice, equipment and facilities necessary for each of the Biosafety Levels (BSL), based on EU Directive 98/81 and WHO Biosafety Manual. This information is summarized in a general way in Table 5. A more comprehensive listing of all requirements and equipment necessary for the four biosafety levels is presented in Table 6. This list is adopted from the WHO Biosafety Manual (Anon, 2004).

At BSL 1 safety is mainly achieved by applying good microbiological techniques. To achieve a higher degree of containment and thus a higher degree of protection against LAIs, the number of requirements increases up to the maximum containment at BSL 4. Besides equipment there are several codes of practice for laboratory access, personal protection and working procedures. Scientific evidence for the

efficiency of these measures is scarce, and most of these measures have been developed based on a long history of microbiological practice and common sense. In Table 7 the codes of practice for BSL 1 and 2 are listed. Table 8 describes the codes of practice regarding access and personal protection for BSL 3 level laboratories.

In the Netherlands the biosafety levels 1 to 4 are called MLI – IV for work with GMOs. These are based on the EU Directives 90/219 and 98/81 and implemented in national regulations (VROM 2004). Many technical requirements and access and personal protection rules are similar, but a major difference is that according to Dutch regulations at MLII level (BSL-2) a biosafety cabinet class II is not optional **but required**. Another difference is that the Dutch regulations provide more detail with respect to procedures. For instance it is stated: "*prepare your work carefully limiting the necessarily movement from one place to another during the microbiological work. Collect all material and equipment before you start and arrange them in an orderly fashion*".

The first level-4 high containment laboratories were built in the 1970s. Until then, researchers had handled extremely hazardous biological agents in so-called glove boxes: hermetically sealed, transparent cabinets fitted with rubber gloves, compatible with biosafety cabinet class III. To day, most level-4 high containment laboratories operate as suit laboratories, where researchers wear full-body positive pressure ("space") suits (Wilson and Chosewood 2007, and EU Directives 90/218/EC, 98/81/EC and 2000/54/EC). Key features of level-4 high-containment laboratories are the safeguards to prevent failure and faults of containment systems and measures. There is thus redundancy of critical systems and biosafety procedures.

Although the objectives that the legislator want to reach using containment levels and procedures are not always explicitly mentioned, one could deduce some guiding objectives, as we have summarized in Table 9.

Table 5. Biosafety levels (BSL), practices, and safety equipment necessary to the four levels of microbial containment

BSL	Practices	Safety equipment	Facilities
1	Standard microbiological practices	None required	Open bench top and sink*[1]
2	BSL-1 plus biosafety warning biosafety manual, waste decontamination and medical surveillance policy	Class I or II BSC laboratory coats gloves, face protection face mask optional	BSL1 plus autoclave
3	BSL-2 practice plus controlled access, decontamination of waste and laboratory clothing, baseline serum sample	Class I or II*[2] BSC protective clothing gloves, respiratory protection optional	BSL-2 plus physical separation from corridors, self-closing double doors, no air recirculation, negative airflow in laboratory, HEPA-filtered air outlet*[2]
4	BSL-3 practice plus clothing change before entering shower on exit, all material decontaminated on exit	Class III BSC or class I or II BSC in combination with full-body, air-supplied positive-pressure suits	BSL-3 plus separated building or isolated zone dedicated supply and vacuum and decontami-nation systems plus air filtration.

Based on EU Directive 98/81 and WHO Biosafety Manual (Anon. 2004, Nulens and Vos, 2002).

*[1] According to EU Directive 98/81 an autoclave is required for ML- I.

*[2] According to EU Directive 98/81.

Table 6. Requirements for the equipment and design for laboratories of different levels of biosafety

	Biosafety level			
	1	2	3	4
Isolation of laboratory	No	No	Yes	Yes
Rooms sealable for decontamination	No	No	Yes	Yes
Ventilation:				
- inward air flow	No	Desirable	Yes	Yes
- controlled vent. system	No	Desirable	Yes	No
- HEPA filtered exhaust	No	No	Yes*[1]/No*[2]	Yes
Double door entry	No	No	Yes	Yes
Airlock	No	No	No	Yes
Airlock with shower	No	No	No	Yes
Anteroom	No	No	Yes	-
Anteroom with shower	No	No	Yes/No*[3]	No
Effluent treatment	No	No	Yes/No*[3]	Yes
Autoclave:				
- on site	Yes*[1]/No	Yes*[1]/Desirable	Yes	Yes
- in laboratory	No	No	Desirable	Yes
- double ended	No	No	Desirable	Yes
Biological Safety Cabinets	No	Yes*[1]/Desirable	Yes	Yes
Personnel safety monitoring capability	No	No	Desirable	Yes

After WHO Laboratory Biosafety Manual, Anon. 2004.
*[1] according to Dutch regulations (VROM 2004).
*[2] dependent on location of exhaust.
*[3] depending on agents used.

Table 7. Codes of practice regarding access and personal protection for BSL 1 and 2 level laboratories

Acces

- The international biohazard sign must be displayed on doors of Laboratories in which microorganisms of risk group 2 or higher are handled
- Only authorized personnel is allowed to enter the laboratories
- Laboratory doors should be kept closed
- Children under 16 are not allowed to enter
- Access to animal houses should be specially authorized
- Animals not involved in the work are not allowed
- Signs with no smoking, no drinking no eating should be displayed in and outside the laboratory (not required in some countries, incl. The Netherlands)

Personal protection

- Laboratory coveralls, gowns or uniforms must be worn in the laboratory
- Appropriate gloves must be worn during all work involving contact with infected material, after use gloves should removed aseptically and hands must be washed
- Personnel must wash their hands after handling infectious material or animals and before they leave the laboratory
- Safety glasses of face shields must worn when it is necessary to protect the eyes and face from splashes, impacting objects of artificial UV radiation
- It is prohibited to wear protective clothing outside the laboratory
- Open toad footwear should not be worn
- Eating, drinking and applying cosmetics and handling contact lenses is prohibited in the laboratory
- Storing human foods or drinks in the laboratory is prohibited
- Protective clothing should not be stored in the same lockers as street clothing.

Adopted form the WHO Biosafety Manual (Anon, 2004).

Table 8. Codes of practice regarding access and personal protection for BSL 3 level laboratories

Acces

- The international biohazard warning symbol and sign displayed on laboratory access door must identify the biosafety level and the name of the laboratory supervisor who controls access, and indicate any special conditions for entry into the are, e.g .immunization.

Personal protection

- Laboratory protective clothing must be of the type with solid-front or wrap-around gowns, scrub suits, coveralls, head covering and, where appropriate, shoe covers or dedicated shoes. Front-buttoned standard laboratory coats are unsuitable, as are sleeves that do not fully cover the forearms. Laboratory protective clothing must not be worn outside the laboratory, and it must be decontaminated before it is laundered. The removal of street clothing and change into dedicated laboratory clothing may be warranted when working with certain agents (agricultural or zoonotic agents).
- Open manipulations of all potentially infectious material must be conducted within a biological safety cabinet or other primary containment device.
- Respiratory protective equipment may be necessary for some laboratory procedures or working with animals infected with certain pathogens.

Adopted form the WHO Biosafety Manual (Anon, 2004). Note that the codes of practice for BSL 1 and 2 levels (Table 7) apply as well, except when modified as described above.

Table 9. Objectives of microbiological containment rules

	level 1	level 2	level 3	level 4
Reduction of direct and oro-fecal transmission of non-pathogenic microorganisms to lab personnel; reduction of transmission of non-pathogenic microorganisms outside the laboratory; general hygiene	+	+	+	
Reduction oro-fecal transmission of enteric pathogens to lab personnel		+	+	
Reduction of airborne transmission of pathogenic microorganisms to lab personnel		+	++	
Reduction of transmission of pathogenic microorganisms outside the laboratory by direct contact, environmental and airborne spread		+	++	
Strict prevention of transmission of very virulent microorganisms to lab personnel				+++
Strict prevention of transmission of very virulent microorganism outside the laboratory; calamity procedures				+++

This table specifies objectives that may be achieved by containment measures at BSL levels 1 – 4, as specified by EU 90/679 and VROM. At increasing safety levels,

there are additional demands regarding equipment and procedures while those pf the lower levels are maintained. At level 1 equipment and procedures are directed at providing general hygiene, which probably mainly diminish infection by the oro-fecal route. At level 2 a BSC class II is optional or required as in the Netherlands, providing mainly respiratory protection to the laboratory workers; environmental spread is diminished by keeping windows closed. At level 3 further environmental protection is provided by a sluice, disinfection facilities, and a unidirectional HEPA-filtered air flow. At level 4 all procedures and equipment are directed towards preventing any microbial transmission.

5. Approaches of biosafety evaluation

The scientific literature on evaluation of effectiveness of biosafety measures is very scarce and does not provide a consensus approach. Effectiveness of biosafety measures may be evaluated by different approaches and at different levels. In Table 10 we have given a simple classification of approaches that collectively may provide guidance in evaluation activities. This table presents questions and purposes of single evaluation activities. A first level of evaluation may be directed at measuring the effectiveness of single containment equipment and procedures, such as the filtering capacity of face masks and safety cabinets under experimental circumstances. Such evaluation could be directed at physical or, preferably, microbiological criteria. Subsequently these single apparatus and procedures should be evaluated during practical work. Evidently by taking this step from experimental challenge to practical work, unforeseen circumstances that may occur during practical work may be detected. For example, turbulences caused by movements of personnel during practical work may lower the protection afforded by biosafety cabinets. Air leakage may occur along respiratory masks during work. Masks may not fit perfectly.

A subsequent level of evaluation would be the laboratory as a whole, including its design and construction, the equipment, and working instructions. Again such an evaluation can be done experimentally during a validation of the laboratory process, or actually in a working laboratory setting. An experimental approach may use the deliberate release of indicator particles or model microorganisms. Evaluation of laboratory safety under field circumstances may include analysis of environmental samples taken inside and near the laboratory. In this case the effectiveness of biosafety measures and working instructions are actually evaluated during practical work and includes compliance of workers to working instructions, their experience and training, unintentional incidents, and efficacy of containment measures.

Finally one may evaluate the effectiveness of measures at the clinical-epidemiological level, examining the overall effectiveness of measures in their capacity to prevent infection of laboratory workers and others. Evidently laboratory workers play a central role in such an evaluation as they are both the persons that are at high risk and that may pass infections to others. Such epidemiological studies may follow a passive or an active searching approach. Evidently, for ethical reasons this level of evaluation is usually not suitable for an experimental approach.

Table 10. Approaches of biosafety evaluation

	Experimental approach	**Practical conditions**
Equipment and procedures	*Do single devices and procedures function effectively upon experimental challenge with particles or model microorganisms?*	*Do single devices and procedures function effectively during practical work?*
Laboratory	*Does the laboratory as a whole afford effective containment upon experimental challenge with particles or model microorganisms?*	*Does the laboratory as a whole afford effective containment during practical work?*
Laboratory workers and environment	*N.A.*	*Are laboratory workers and the environment protected against infection?*

Classification of approaches to evaluate containment measures. Examples of the major questions and purposes of the evaluation steps are given in italics. N.A.: not applicable.

5.1 Compliance to procedures and training

We consider optimizing the training of personnel and monitoring their compliance to procedures important, as the best biosafety measures are as good as the participation and discipline of laboratory workers themselves. In many reports extensive training of laboratory workers and the proper execution of guidelines is mentioned as one of the most important measures to prevent incidents (Lucero and Sinerez, 2005). However, poor compliance has also been reported (Gershon et al 1995, Vaquero et al 2003). In Argentina incidences with pathogenic microorganisms were reduced after setting up a training program and providing protocols (Lucero and Sinerez, 2005). The importance of training was also emphasized by experiences during the SARS outbreak in 2003 (Lim and Tsang 2006, Normile 2003). Laboratory escapes of the virus occurred in Singapore, Taiwan and Beijing from BSL3 laboratories because of breaches in good laboratory practice rather than failure of the facilities. Extensive contamination occurred because gloves were inappropriately worn and contaminated surfaces were not disinfected (Lim and Tsang, 2006).

6. Experimental and observational data on the effectiveness of containment measures

6.1. Do single devices and procedures function effectively?

Because most infections in the laboratory occur via aerosols, infected material and surfaces (Schellekens, 2001), equipment directed at minimizing air-borne infections received most attention. There are three classes of BSCs with different levels of protection, class I, II and III (Anon, 2004). In addition, within BSC II there are 3 subtypes. Class II safety cabinets consist of a chamber with a small open front in which an airflow is generated to prevent microorganisms from escaping the chamber. Laboratory workers sitting behind the cabinet insert the hands and arms into the chamber. All objects and the arms of the worker can disturb the airflow and cause the microorganisms to escape. Class III biosafety cabinets are basically similar, but the front is completely closed and can be accessed via attached gloves.

Safety cabinets should meet legal standards as, for example, defined by the European Union (EN12469). BSCs have been improved significantly during recent years, amongst others due to this EN12469 standard. Unfortunately, literature of the containment efficiency of class II BSCs is scarce, mainly addressed in older publications, and virtually absent of class III BSCs.

In general BSCs provide a good level of protection when operated and maintained correctly (Osborne et al 1999). However, in several older studies, before the introduction of EN12469, it was shown that personnel working with open front safety cabinets can still be exposed to infectious doses of microorganisms (Barbeito and Taylor 1968, Kruse 1962, Philips 1965). Barbeito and Taylor (1968) investigated the efficiency of containment of a BSC under three different closure conditions and different air velocities. In the cabinet between 10^5 and 10^6 microorganisms per cubic foot were released in 5 minutes. When the glove panel was removed, a human infectious dose was released and the number of microorganisms that escaped containment increased with decreased air velocity. Moreover, an increase in human activity in the cabinet resulted in increased numbers of microorganisms escaping the cabinet. When the glove panel was attached, no microorganisms could be detected outside the cabinet. A remarkable finding was that when the glove panel was installed without the gloves attached no microorganisms escaped form the cabinet. Their main conclusion was that laboratory workers are only protected from infectious microorganisms when they use closed safety cabinets with high airflow velocities, and that the effectiveness of biosafety cabinets is compromised by the activity of the workers. Macher and First (1984) performed measurements on exposure of workers

to bacterial spores using a class II BSC with an adjustable work opening. Inside the cabinet aerosols of bacterial spores were created and the escaping spores were measured. Work opening height appeared to be a significant predictor of spore concentrations outside the BSC (Macher and First, 1984). Similarly, air flow velocity was negatively correlated with the concentration of escaping spores. Human activity in the cabinet, such as hands moving through the opening, also resulted in the escape of spores. Spore concentrations in the operators breathing zone were about 24 times higher than acceptable levels. Surprisingly, working in the rear of the cabinet was less safe than working in the front since the close proximity of the body to the cabinet influenced the airflow. Thus it is essential for safe working conditions to limit the movement of arms and hands by arranging the equipment in the most practical way. Heidt (1982) also tested the efficiency of a class II BSC. This author concluded that the cabinet provided sufficient protection since microorganisms only escaped at the highest densities of the test aerosol created inside the cabinet. Since the number of bacteria detected was very low, this was considered to be acceptable. Osborne and co-workers (1999) investigated a number of BSCs and calculated the Operator Protection Factors (OPFs), as assessed by still and latterly limited 'in-use' KI-Discus tests. OPF is defined as the ratio of exposure to airborne contamination generated on the open bench to the exposure resulting from the same dispersal of airborne contamination generated within the cabinet (Kennedy and Collins 2000). Most BSCs had OPFs higher than 100.000, except when room pressure changed or when draughts occurred in the laboratory. Cabinet performance of class II cabinet was shown to be affected by the movements of the worker, and some movements reduced OPF results as found before by Macher and First (1984). However, the levels of failure were marginal. The OPF tests revealed that a selected class II unit provided the same OPF as a class I unit when properly used.

Although literature is not unequivocal, it appears that the use of BSCs decreases LAIs significantly (Heidt 1982, Macher and First 1984, Osborne et al 1999, Rusnak et al 2004). However, in a recent publication Rusnak et al (2004) examined illness surveillance data archived from the US offensive biological warfare program (from 1943 to 1969) and concluded that BSCs and other measures failed to sufficiently prevent illness from agents with lower infective doses in a high-risk research setting.

Though required in some countries (incl. The Netherlands), cabinet performance is not generally assessed. However, on-site containment tests indicated that 37 class II safety cabinets (all with adequate type test certification and including 18 new installations) failed to meet the OPF requirements as defined in BS 7526. Thus

testing for containment using an OPF test appears essential both at commissioning and during routine maintenance (Clark 1997, Osborne et al., 1999).

While formaldehyde gas has been used for over 100 years for decontamination, the efficacy of this process remains controversial (Munro et al 1999). Moreover because of its toxicity the use of formaldehyde requires containment procedures in itself (Kennedy and Collins 2000). Formaldehyde decontamination of BSCs is usually validated using spore strips and culture. Therefore poliovirus, *Mycobacterium bovis* strain BCG, or *Bacillus* spores have been used. Bacterial spores on stainless steel appear resistant to decontamination, and using bacterial spores to validate decontamination is too slow. Therefore commercial biological indicator tests have been developed, which may be an aid in detecting incomplete decontamination. Difficulties in obtaining effective decontamination by using formaldehyde gas have been demonstrated. Factors contributing to the effectiveness of decontamination by formaldehyde range from the formaldehyde level, the relative humidity, the temperature levels, and the medium to be contaminated. Locations beyond the exhaust filters of BSCs were the most difficult to decontaminate.

Modern cell sorting equipment has become an important tool in microbiological laboratories (Lennartz et al 2005, Perfetto et al 2003). Because cell sorters lead to aerosol formation and are not easily accommodated by regular BSCs, this kind of apparatus could cause the operators to become contaminated (Lennartz et al 2005, Perfetto et al 2003, Schmid et al 2003). In addition, their high costs often prohibit their incorporation within BSL facilities. To solve this problem Lennartz and co-workers (2005) integrated a FACS-sorter into a specially developed class II BSC. Biosafety was subsequently tested by using T4 bacteriophage aerosols and shown to be excellent. Bacteriophages were readily detected in and outside when the airflow of the BSC was off, but when the BSC was turned on no bacteriophages could be detected outside (Lennartz et al 2005). Many FACS-protocols include inactivation steps, including the use of fixatives based on alcohols or formaldehyde. Some of these protocols have been evaluated for antimicrobial activity directed against specific pathogens, in particular HIV. Formaldehyde at concentrations of 0.5 to 2 % is effective in inactivating HIV, but the ability of fixatives to inactivate other microorganisms in FACS equipment, including hepatitis B virus, has not been demonstrated. In addition some protocols employ non-fixed cells. While analytic cytometers are engineered not to produce aerosols, jet-in-air cell sorters generate droplets and microdroplets that may be aerosolized. Recently high-speed cell sorting using high operating pressures with an increased potential for aerosol generation and an enhanced risk of sample splashes at the sample introduction port has become

more prevalent. At the same time, instrument manufacturers have become more safety conscious, developed novel devices for containment of aerosols and splashes, modified sample uptake ports on cell sorters, and installed mechanisms to stop sample flow in case of a nozzle clog to reduce operator risks (Schmid et al 2003, Perfetto et al 2004). A FACS Vantage cell sorter was thus modified for safe use with potentially HIV-infected cells. Safety tests with bacteriophages were performed to evaluate the potential spread of biologically active material during cell sorting. The bacteriophage sorting showed that the biologically active material was confined to the sorting chamber. A failure mode simulating a nozzle blockage resulted in detectable droplets inside the sorting chamber, but no droplets could be detected when an additional air suction from the sorting chamber had been put on (Sørensen et al 1999). While these observations may be reassuring, some recommendations regarding the use of FACS equipment are important (Schmid et al 1999, 2003). Training has to include performing aerosol containment testing of instruments to be used for biohazardous sorting. In addition waste fluid has to be collected in 10 % sodium hypochlorite, and fluid lines should be disinfected using a 1 : 10 dilution of 5.25 % sodium hypochlorite. Notwithstanding their potential hazards, no documented disease transmission through the use of a cytometer has occurred (Schmid et al 1999, 2003).

A few papers examined the efficacy of face respirators and surgical masks. For example, Balazy et al (2006) examined the performance of 2 types of N95 half-mask, filtering face piece respirators and 2 types of surgical masks. The collection efficiency of these respiratory protection devices was investigated using MS2 virus (a nonharmful simulant of several pathogens) in a particle size range of 10 to 80 nm. Penetration of virions through N95 respirators - certified by the National Institute for Occupational Safety and Health (NIOSH) - can exceed an expected level of 5%. The tested surgical masks showed a much higher particle penetration of the MS2 virions: 20.5% and 84.5%.

6.2. Does the laboratory as a whole afford effective containment?

The proper functioning of equipment should not only be evaluated in isolation but also in the context of the entire laboratory. In considering biosafety of laboratories, the proper functioning of autoclaves may be overlooked. Barbeito and Brookey (1976) and Marshall et al (1999) emphasize the potential of autoclaves to release viable microorganisms into the atmosphere, and emphasize the importance of proper sterilizer location, ventilation, containment of heavily contaminated loads, and adequate sterilizer maintenance.

One of the few studies to assess contamination of the laboratory environment with pathogens found in blood examined 800 environmental samples taken from 10 clinical and research laboratories working at BSL-2 level at the National Institutes of Health (NIH, US). Thirty-one samples from 11 work stations in three laboratories contained hepatitis B virus surface antigen (HbsAg). Factors associated with environmental contamination included flawed laboratory techniques (mouth-pipetting, splashing, placing pens in the mouth, improper use of equipment, and improper instrument design requiring external wash steps) (odds ratio [OR] 9.78, 95 % confidence interval [CI] 1.46, 65.49), high work loads (OR 5.06, 95 % CI 0.8, 31.96), and inappropriate behaviors (including not wearing gloves) (OR 2.75, 95 % CI 0.44, 17.4). Flow cytometry was identified as technique with the most frequent occurrence of overt spills (Evans et al 1990). Indeed hepatitis B virus (HBV) infection was among the most commonly reported LAIs. Laboratory workers in urban medical centers may have been at almost three times the risk of acquiring HBV infection than other hospital employees due to exposure to patients' blood, and 7 to 10 times the risk than that of the general public (Evans et al 1990).

Some evidence of the effectiveness of a BSL-3 laboratory environment may be derived from experiences with a specially designed BSL-3 laboratory for autopsies of patients with severe acute respiratory syndrome (SARS) (Li et al 2005). SARS coronavirus is highly infectious, and during the outbreak of SARS more than 30% of the approximately 8000 infected persons were health care workers. The autopsy laboratory was established in Beijing Ditan Hospital (which was designated the SARS hospital during the outbreak of SARS in China) in May 2003. Remarkably, the efficiency of decontamination in this laboratory was evaluated by a sarin simulant test. Therefore a sarin simulant aerosol of 0.3 μm particles at 4 mg/L was generated and spread by a special device in the contaminated area. Sarin could not be detected in either the semicontaminated area or clean area, and particles >0.3 μm in size were not detected in the exhaust air. Twenty-three pathologists and technicians participated in sixteen complete autopsies that were performed on patients with clinically confirmed or suspected SARS, of which seven cases were later confirmed to be SARS infections. None of these personnel demonstrated any evidence of SARS infection.

The set of biocontainment measures that define level-4 biosafety is comfortably the most comprehensive and stringent, but each setting and laboratory design is unique and comparative data on their containment effectiveness is non-existent. A problem in assessing the effectiveness of level-4 containment measures, is that isolated containment measures are considered insufficient. Thus individual components like

autoclaves, incinerator, chemical decontamination shower, gaseous decontamination systems, air ventilation systems and HEPA filters can be tested for physical parameters during normal operation and under extreme conditions, but it remains unclear how closely the simulated test conditions resemble the real-life situation. Effectiveness of HEPA filters is typically validated using bacterial spore strips or particles.

6.3. Are laboratory workers and the environment protected against infection?

The analysis of laboratory accidents may illustrate what can go wrong and point the way to improvements. Such accidents are one of the most relevant parameters to evaluate the overall effectiveness of integrated biosafety measures. However, the epidemiology of the incidence and severity of LAIs is largely unknown as there are neither national surveillance and monitoring systems with complete coverage, nor many systematic studies on their occurrence (Kahn 2004, Sewell 1995). Denominator data that are necessary to calculate the actual incidence of LAIs are usually lacking. In addition LAIs may be subclinical, may have an atypical incubation period and route of infection, and laboratory workers and directors may be reluctant to report them because of fear of reprisal and stigma (Sewell 1995, Harding and Byers 2006). Therefore much information is obtained from anecdotal case reports and some retrospective questionnaires. Such case reports do not always report on possible failure of biosafety procedures or unintended accidents. While accidental parenteral inoculation of infectious material appears one of the leading causes of LAIs, most LAIs appear to occur even with the best safety precautions in place (Pike 1979, Sewell 1995). A summary of some recent LAIs is given as annex in Table 11.

6.3.1. *Reviews*

Most LAIs are caused by microorganisms that are very pathogenic or that need a very low infectious dose, including arboviruses, Venezuelan equine encephalitis, hantavirus, hepatitis B virus, hepatitis C virus, typhus, *Brucella sp., Coxiella burnetii, Francisella tularensis, Mycobacterium tuberculosis, Salmonella sp., Shigella sp., Chlamydia psittaci,* streptococcal infections, histoplasmosis, leptospirosis, tularemia, *Blastomyces dermatitidis, Coccidioides immitis, Cryptosporidium sp.,* coccidiomycosis, and dermatomycosis (Harding and Byers 2006, Pike 1979, Sewell 1995, Wedum 1961). A direct link to accidents or exposure events, such as aspiration, injection, cut, spill or bite, appears only apparent in a minority of the LAIs, while the majority is likely caused by undefined exposure to aerosols (Harding and

Byers 2006, Pike 1979, Wedum 1961, 1964a, 1964b, Yagupsky et al 2000). Indeed aerosols have been responsible for major outbreaks of LAIs caused by *Brucella* spp., *Coxiella burnetti* (Q fever), *Chlamydia psittaci* (psittacosis), and *M. tuberculosis.* The main hazards for inoculation (Sewell 1995, Pike 1976, Pike 1979) include 1) parenteral inoculation, 2) inhalation of infectious aerosols, and less common, 3) accidental oral ingestion, and 4) direct contact with mucous membranes or (broken) skin. Special hazards occur when working with infected animals. The presence of highly pathogenic microorganisms in unknown clinical samples likely explains the high incidence of tuberculosis among laboratory workers. In different studies the incidence of tuberculosis in laboratory personnel is estimated to be three to 100 times the frequency observed in the general population. The high infectivity of *M. tuberculosis* is related to its low infective dose (i.e. a 50 % infective dose of < 10 bacilli) (Richmond et al 1996). Schellekens (2001) calculated that 1 out of a 100 to a 1000 laboratory workers per year is infected, but recent studies suggest that the rate of LAIs per person per year is decreasing (Osborne et al 1999, Wilson and Chosewood 2007, Schellekens 2001).

Pike (1979) tabulated the most common sources of LAIs from published literature and survey data. In the period 1924-1977 there were 4,079 reported cases of LAIs with 168 casualties. In the subsequent period 1980 – 1991 the number of reported cases was 375 with 5 casualties. At the time of Pike's survey most LAIs (59%) occurred in research laboratories, compared with 17% in diagnostic laboratories. The highest mortality rate (7.8 %) was associated with psittacosis. At that time, approximately 70% of LAIs resulted from work with the infectious agents (21%) or animals (17%), exposure to aerosols (13%), and accidents (18%). Less frequent sources of infection included clinical specimens (7%), autopsies (2%), and contaminated glassware (1%). Most causes of LAI were unknown (82%), and only in 18% of the reported cases the cause could be attributed to accidents, associated with the use of sharps such as needles (25%), injuries by glass (16%), splashes or spills (27%), mouth pipetting (13%), and bites by laboratory animals (14%). Many of the LAIs of unknown origin were likely caused by exposure to an infectious aerosol.

A recent survey of symptomatic and asymptomatic LAIs has been conducted by Harding and Byers (2006), who reviewed 270 publications from 1979 to 2004, a period during which much has been done to improve laboratory safety while the work load in laboratories increased. A decrease in the number of LAIs would therefore be expected, which however needs knowledge on the total population at risk and the total number of infections. Harding and Byers (2006) found a total of 1,448 cases and 36 deaths, 6 of which were aborted fetuses. The infections occurred in clinical,

research, teaching, public health, and production facility laboratories, with clinical and research laboratories accounting for approximately 76 %. In recent years more LAIs from clinical laboratories were reported, probably due to a more active employee health program, the absence of biosafety containment equipment in a number of clinical laboratories, or the fact that during the early stages of culture identification, personnel are working with unknowns and may not be using adequate containment procedures. Like earlier findings the authors report that only a small proportion of the LAIs resulted from actual accidents. Most were acquired by simply working in the laboratory or by exposure to infected animals.

Sewell (1995) concluded that adherence to the guidelines promulgated by the various regulatory agencies decreases the risk of occupational exposure to infectious agents. However, he also recommended additional studies to evaluate the effectiveness of other safety measures implemented or mandated in the laboratory. Interestingly, Sewell (1995) describes personal risk factors of laboratory workers associated with accidental infections. Characteristics of persons who have few accidents include adherence to safety regulations, a respect for infectious agents, ‘‘defensive’’ work habits, and the ability to recognize a potentially hazardous situation. In contrast, persons involved in laboratory accidents tend to have low opinions of safety programs, to take excessive risks, to work too fast, and to be less aware of the infectious risks of the agents they are handling. Also, men and younger employees (17 to 24 years old) are involved in more accidents than women and older employees (45 to 64 years old).

While many reports emphasize the importance of personal protection, there are indications that extensive personal protection by double gloves, face masks and protective clothing is not the sole solution, since such measure can reduce dexterity of the laboratory worker leading to increased accidents (Sawyer et al., 2007). This indicates that the use of sharps should be minimized when workers wear extensive personal protection.

Laboratory-acquired parasitic infections, both protozoa and helminths, have been extensively reviewed by Herwaldt (2001). Important, because protozoa, in contrast to most helminths, multiply in humans, even a small inoculum can cause illness. The author summarizes 199 case reports in laboratory and health care workers. The most frequently reported parasitic infections were caused by *Trypanosoma cruzi*, *Toxoplasma gondii*, *Plasmodium spp, Leishmania spp.,* and *Cryptosporidium parvum*. Two cases (one of Chagas’ disease and one of toxoplasmosis) were fatal. However, as with other infections, accurate counts of accidental exposures and infections and information on the risk per person-year are unavailable. Some of the

laboratory-acquired parasitic infections were directly linked to accidents (bite by an escaped infected mosquito) and poor laboratory practices, such as recapping a needle, removing a syringe from a needle, working barehanded, mouth pipetting, and working too fast. From 105 cases an accident or a likely route of exposure could be presumed, 47 (44.8 %) of which had a percutaneous exposure via a sharp object. In other cases no apparent accidents were recognized or reported, suggesting that subtle exposures (e.g., contamination of unrecognized microabrasions and exposure through aerosolization or droplet spread) resulted in infection.

6.3.2. *Surveys*

Walker and Campbell (1999) did one of the few systematic, but retrospective studies. They carried out a retrospective questionnaire survey of 397 responding UK laboratories covering 1994 and 1995. Approximately 75 % of these were diagnostic laboratories. 14% of those who responded were research laboratories, and 9% were teaching laboratories. Over 55,000 personyears of occupational exposure were covered, and only nine cases of LAI were identified, giving an infection incidence rate overall of 16.2/100 000 person-years, compared with 82.7 infections/100 000 person-years found in a similar survey covering 1988 and 1989, which was conducted by Grist and Ernslie (1989). This decline in incidence continues the trend previously reported for the period 1970–1989. Infections were commonest in females (in contrast to the findings reported by Sewell 1995), in relatively young staff, in microbiology laboratory workers, and in scientific/technical employees. Gastrointestinal infections predominated, particularly shigellosis, but few specific etiological factors relating to working practices were identified. These included a broken glass leading to a hand cut, a rat bite, and aerosol contamination. In most cases no clear accident was reported. Lack of experience was cited as a definite factor in two of the cases. Single cases of hepatitis C, *E. Coli* O157, and *M. tuberculosis* infection were identified, in addition to single cases of non-specified septicemia and gastroenteritis. The absence of any cases of hepatitis B infection, as in 1988–1989, reflects a sharp decline since 1970 and was ascribed to increased awareness, better technique, and the availability of immunization. Furthermore, the absence of eye infections and the paucity of skin infections may indicate good technique and use of protective equipment. Despite the shortcoming of this study (retrospective study design, no reliable denominator, potential under-reporting or under-recording, no detection of asymptomatic infections), the authors concluded that the small number of cases identified indicates high standards of infection control, although they still recognized room for improvement. Finally, the study emphasized

that the notification system in place in the UK to report LAIs is inadequate to the task of monitoring their true incidence in a comprehensive way, a conclusion that probably holds true for the situation in many other countries. For comprehensive monitoring of the incidence of LAIs, it is needed to establish a routine, active surveillance program or prospective survey, which has the support and commitment of the laboratories themselves.

Recently, a report was drafted on the biosafety status of clinical laboratories in Japan (Yamashita et al., 2007). Data were obtained from 431 hospitals and 301 institutions. The authors found 28 cases of possible laboratory-associated tuberculosis infection of which 25 could be associated with the lack of BSCs, which are required for work with *M. tuberculosis*. Other risk factors were insufficiently skilled equipment operation and rupture accidents during centrifugation of blood. Within the last 5 years 1,534 events of self inflicted needle punctures were recorded (Yamashita et al. 2007).

A retrospective survey of incidents occurring during biotechnological and clinical work in Flanders, Belgium, indicated that on average 13.6 incidents occurred per year among 7,302 laboratory workers. As a result, 69 persons (< 1 %) were exposed to biological agents, resulting in 2 LAIs, caused respectively by *L. monocytogenes* and *B. melitensis*. Most incidents occurred in clinical laboratories, likely caused by the higher number of working hours actually spent in clinical laboratories and the sometimes unknown nature of microorganisms. Handling of experimental animals and waste were considered as risky. Most incidents were caused by human failure and included prick accidents, spilling, breaking, and maintenance work carried out in the laboratory (De Cock en Van Eetvelde 2007).

Sejvar et al (2005) undertook a systematic, retrospective evaluation of the risk of meningococcal disease among clinical microbiologists and an assessment of the laboratory procedures that might predispose technicians to infection. Cases of suspected or proven laboratory-acquired meningococcal disease were identified by placing an information request on e-mail discussion groups of infectious disease, microbiology, and infection control professional organizations. Sixteen cases of probable laboratory-acquired meningococcal disease occurring worldwide between 1985 and 2001 were identified, including six US cases between 1996 and 2000. Nine cases (56 %) were serogroup B; seven (44 %) were serogroup C. Eight cases (50 %) were fatal. In 15 cases (94 %), isolate manipulation was performed without respiratory protection. An average of three microbiologists are estimated to be exposed to the 3,000 meningococcal isolates seen in US laboratories yearly resulting in an attack rate of 13/100,000 microbiologists between 1996 and 2001, compared to 0.2/100,000 among US adults in general. The case/fatality rate of 50 % seen among

survey cases is substantially higher than that observed among community-acquired cases, which may be explained by ascertainment bias due to underreporting of mild cases of disease. However, an alternative possibility is that clinical microbiologists routinely work with highly virulent strains and high concentrations of organisms. All cases identified in this inquiry occurred among microbiologists and not among workers in other areas of the clinical laboratory. This suggests that exposure to isolates of *N. meningitidis*, and not patient samples, represents the increased risk for infection. In addition, all isolates were derived from sterile sites. None of the microbiologists identified were working with isolates obtained from pharyngeal or respiratory secretions, suggesting that such pharyngeal isolates represent a lower risk, presumably due to their lower pathogenicity. The authors concluded that prevention should focus on the implementation of class II BSCs or additional respiratory protection during manipulation of suspected meningococcal isolates. Following two cases that prompted this survey, CDC has instituted a prospective surveillance for laboratory-acquired meningococcal disease (CDC 2002).

In the Netherlands two surveillance systems monitor the occurrence of labour-acquired infections, i.e. not exclusively LAIs. The number of reported LAIs is low, but both systems suffer from serious underreporting and do not provide details of transmission route or accidental cause of infection (Heimeriks 2007) .

6.3.3. *GMO-associated laboratory accidents*

Fortunately the number of accidental releases or LAIs with GMOs appears very scarce. Openshaw et al (1991) report an accidental infection of a laboratory worker with recombinant vaccinia virus (Western Reserve strain) expressing proteins of respiratory syncytial virus. The infection occurred through two separate needle accidents during the same work session, although the worker was experienced in the procedure. The procedure was subsequently modified to prevent further accidents. The laboratory worker had been vaccinated with standard smallpox vaccine, a practice that may have restricted the severity of symptoms to local redness and swelling.

Mempel et al (2003) report the case of a recombinant vaccinia infection in a previously vaccinated researcher working with various genetically modified strains. The isolated virus carried a functionally inactivated cytohesin-1 gene of human origin, which impairs leukocyte adhesion by interacting with the LFA/ICAM-1 axis. The immunomodulating nature of the inserted construct might have added to the infectivity of the virus. Although the paper does not detail safety procedures in the lab, the infection occurred while the handling was considered properly. Contact

infections were not reported. The researchers emphasize the necessity of vaccinia vaccination of laboratory workers. In the US and Canada specific recommendations exist for laboratory personnel that conduct research with (recombinant) orthopoxviruses, including vaccinia virus (Williams and Cooper 1993, CDC 2001). The Advisory Committee on Immunization Practices recommends revaccination at least every 10 years for persons working with nonhighly attenuated vaccinia viruses, recombinant viruses developed from nonhighly attenuated vaccinia viruses, or other nonvariola orthopoxviruses. To ensure an increased level of protection against more virulent nonvariola orthopoxviruses (e.g., monkeypox), empiric revaccination every 3 years can be considered. In contrast, mandatory guidelines with respect to vaccinia vaccination do not exist in Europe (Isaacs 2004).

Lewis et al (2006) report a case of ocular vaccinia infection in an unvaccinated laboratory worker. The infecting virus was a unique form of recombinant Western Reserve vaccinia virus constructed in the research laboratory. Although laboratory staff generally followed established biosafety precautions, several opportunities for virus exposure were identified. Experiment were performed partly outside a BSC. Staff infrequently wore eye protection. Laboratory coat sleeves were not elasticized and did not always cover the wrist. Waste pipettes were not disinfected before removal from the BSC. Instances occurred in which samples with low titers of live virus were removed from the BSC, transported to other parts of the facility, and manipulated. In addition, laboratory staff routinely vortexed tubes containing live virus outside the BSC.

Another case of laboratory-acquired vaccinia infection (presumably non-recombinant) was reported by Wlodaver et al (2004). This infection occurred in a laboratory technician who had not been previously vaccinated and who developed generalized vaccinia. She had accidentally cut a finger on a cover slip while working with vaccinia virus. Evaluation of this accident in her laboratory prompted to a review of procedures for handling contaminated glassware.

Moussatché et al (2003) report another accidental needle stick inoculation of a laboratory worker with vaccinia virus. Although the patient had previously been vaccinated against smallpox, severe lesions appeared on the fingers.

In conclusion, LAIs with GMOs appear very seldom and appear to be restricted to infections with recombinant vaccinia virus. Although laboratory accidents with other GMOs may have been unnoticed due to their subclinical course, this situation seems to reflect that vaccinia is very widely used. Perhaps more important, the recombinant virus is still pathogenic, and this might be enhanced by certain gene inserts. Vaccinia virus infection can be established via several routes, including breaks in the skin, and

the infectious dose is probably low. Guidelines for working safely with vaccinia virus, which include vaccination, are available (Isaacs 2004). We consider it nonetheless advisable to work with the highly attenuated strains of vaccinia virus (MVA, NYVAC), or with avian poxviruses that have a restricted host range and do not replicate in mammals (ALVAC, and TROVAC), wherever possible.

6.3.4. *Accidents with risk category-4 organisms*

LAIs with category-4 biological agents (Filoviruses, Arenaviruses, Flaviviruses and Bunyaviruses) are extremely rare and usually occurred earlier in settings with lower levels of biocontainment and/or involved animal work (Feldman et al 2003, Schou et al 2000, Sulkin and Pike 1951ab, Pike 1976, Hanson et al 1976, Miller et al 1987). Rare laboratory incidents with new-world Arenaviruses have been reported in earlier surveys more than 4-5 decades ago (e.g. with Junin virus and Machupo virus) (Hanson et al 1967). Such experiences illustrated the need for more effective measures to reduce hazards.

While this low number of BSL-4 laboratory accidents may be reassuring, the number of BSL4 labs and workers is increasing. This appears in defiance with the "concentration and enclosure" principle, because the risks associated with this work may increase with the number of facilities and workers (Kaiser 2007ab). Concern is further fuelled by several incidents that included unreported infections (a.o. involving *Brucella* and *Coxiella Burnetti*) and other biosafety breaches. In 2006 a Department of Health and Human Services (HHS) Inspector General audit of security procedures found that 11 of 15 institutions had "serious weaknesses" such as unlocked doors and freezers and lax inventory records (Kaiser 2007ab). Another incident in 2007 was the escape of foot-and-mouth disease virus from the Pirbright facilities in the UK, which has been linked to an outdated effluent system and caused several outbreaks of this very contagious disease among cattle and sheep (Health and Safety Executive 2007).

7. Discussion and recommendations

In this paper we have reviewed principles underlying biosafety measures for work with pathogenic microorganisms and GMOs, and we have examined to what extent evidence for their effectiveness is available. Clearly the risks of working with GMOs are considered largely identical to those of pathogenic non-GMO microorganisms, and hence much of the knowledge and containment measures of GMOs are derived from the latter. Regulations appear however more strict for GMOs.

Apparently, amongst many reports on biosafety, we found only scarce information on the evaluation of effectiveness and on criteria to judge effectiveness. We must therewith keep in mind that safety cannot be expressed in absolute terms. It is a relative concept defined in terms of tolerability and acceptability limits (Käppeli and Auberson 1997). This notion implies that workers and regulators try to find a balance between the costs of safety measures and the potential benefits of the work for society. For example, in microbiological work safety measures and associated costs increase from biosafety level 1 towards 4. Indeed, safety measures at levels 1 and 2 are probably insufficient to prevent all infections with microorganisms of the corresponding risk categories, but their consequences at these levels are considered acceptable or negligible.

The current biosafety practice gradually evolved during the previous century. Therefore it is not immediately obvious whether and what principles have been employed to ensure safe work, and on which scientific basis they were build. In this paper we have tried to identify some principles that appear to underlie the current practice. Such principles are clearly partly overlapping and mutually enhance each other. A central activity, either implicit but preferably explicit, is a thorough risk assessment procedure that considers all potentially harmful effects and their possibility of occurrence. Other important underlying principles are the use of – wherever possible and appropriate- biological containment, concentration and enclosure, exposure minimization, physical containment, and hazard minimization.

Clearly, throughout the world regulators have adapted the model of universal precautions based on a classification of microorganisms in four hazard classes and accompanying standard safety practices (Buesching et al 1989, Wilson and Chosewood 2007). The advantage of this model is that work with certain microorganisms can be grouped together to comply with the accompanying containment rules according to their classification. A disadvantage may be that this universal model may overlook the necessity to tailor safety measures on specific microorganisms or specific strains with particular routes of transmission or virulence properties. Therefore, risk assessment remains at the core of any individual

experiment. Such a risk assessment should in particular be based on, preferably quantitative, parameters of transmission, infectivity, and virulence. These should guide the subsequent measures aimed at reducing the amount of microorganisms exposed to individuals to that below a minimal threshold level of infectivity. Nonetheless this universal model of four biohazard classes appears to work well, but we recommend to further harmonize criteria for both non-GMO and GMOs and between different regulatory authorities, such as the EU, WHO, and CDC.

Altogether, the regulations specifying the biosafety containment measures appear to be based on experience, expert judgment, and common sense. They are however not motivated or supported (at least not explicitly) by scientific literature, and often not based on precisely defined or specified properties of microorganisms and vector and insert sequences. In addition, the regulations do not exactly specify the level of protection they aim to afford, for example in terms of diminishing exposure of the laboratory workers below a threshold level of infectivity. Furthermore, it is clear that the physical containment classes 1 to 4 afford increasing levels of containment, but it is not sufficiently clear and scientifically supported to what extent they provide effective protection with regard to prevention of infection of laboratory personnel, prevention of airborne escape, etc. This, together with sometimes not very detailed regulations, puts much responsibility on researchers, lab directors, advisors and regulatory authorities in further detailing working practices. The regulations also do not comprise evaluation procedures to monitor the compliance or effectiveness of the containment provisions. Note that table 12 summarizes our recommendations.

Table 12. Summary of recommendations

- Strengthen the evidence-base wherever possible and feasible in modernizing biosafety measures. This may enhance the effectiveness of biosafety measures as well as compliance with these measures.
- Develop one set of guidelines and regulations for GMOs and non GMOs.
- Develop explicit goals of biosafety measures that can be evaluated.
- Knowledge and measures of biosafety should be directed on, preferably quantitative, parameters of infectivity and transmission.
- Further harmonize biosafety guidelines between regulatory authorities (EU, CDC, WHO).
- Take gene-gene and gene-environment interactions into account in risk assessment.
- Optimize the use of biological containment, in particular with respect to the use of recombinant vaccinia virus.
- Monitor and evaluate biosafety aspects in laboratories and their compliance regularly.
- Optimize systematic surveillance of laboratory accidents and implement recommendations following such accidents. “Blame-free” reporting may enhance the reporting rate. Serological monitoring may support the detection of laboratory infections and should match the risks involved.
- Promote education of laboratory personnel and compliance to the rules.
- Collect data to support the evidence-base of the biosafety practice.
- Develop mathematical models to support the further development of knowledge of biosafety, to detect gaps in our knowledge, and to support the development and evaluation of biosafety measures.

The hazard classification of work with GMOs follows the classification of work with non-GMOs. This extrapolation should be based on a risk estimation as precise as possible considering the genetic modifications involved. In case of doubt or uncertainty of the properties of the GMO involved, regulators and biosafety experts will choose a higher risk classification compared to the risk level of the microorganism from which the GMO has been derived, or they will demand additional safeguards. Basic research of transmission properties of GMOs, in comparison with the non-modified organism, may be helpful in such a risk assessment to further define the risks involved in the manipulation of GMOs. However, often properties, such as infective dose, may be difficult to obtain. In risk assessment we consider it

important to take gene-gene and gene-environment interactions into account, because experience, for example the IL-4/ectromelia construct, has shown that specific gene products may have unwanted effects in a particular environment (Jackson et al 2001).

Regulators do not always require routine evaluation and monitoring of biosafety aspects in laboratories, which we would like to recommend. Routine monitoring of biosafety aspects, including a monitoring of compliance and educational and behavioral aspects, may not easily be implemented, in particular in the many clinical laboratories with their high workload involving a wide variety of sometimes unknown microorganisms, but it may enhance the overall safety awareness. For example, validation experiments using T4 bacteriophage, bacterial spores, or other indicator microorganisms could be useful for this purpose.

From the literature it appears obvious that there is few experience and no consensus on how the effectiveness of biosafety practices should be evaluated. Clearly effectiveness of biosafety measures can be assessed at different levels and under different circumstances that logically complement each other, i.e. one could question whether a single piece of equipment is effective under experimental conditions, or conversely whether the population has not been accidentally exposed to LAIs. Data on the biological containment efficiency of equipment and laboratories is scarce and fragmented, and mainly limited to technical specifications. Monitoring of LAIs therefore appears to play a pivotal role in evaluating the effectiveness of containment and the potential exposure of laboratory workers and the population, but suffers from serious underreporting throughout the world. Many reports of laboratory accidents are anecdotal only. We therefore recommend to optimize the systematic monitoring of laboratory accidents including the serological monitoring of personnel. Infection with microorganisms, either GMO or not, that have a high infective dose or low virulence, usually belonging to risk category 1 and 2, may be difficult to detect. The extent of serological monitoring should therefore depend on the risks involved. A passive sampling strategy, i.e. collecting serum samples at the time of employment and following incidents, may be sufficient for work with low virulent microorganisms, but an active sampling strategy at regular intervals may be considered for class 3 – 4 microorganisms. One clue to optimizing monitoring of accidents may be the introduction of “blame-free” reporting, which aims to share experiences without being punished. In addition to systematic monitoring, retrospective surveys may be very useful, as they may identify certain risk factors as shown for the occurrence of meningococcal disease (Sejvar et al 2005).

Despite the methodological imperfections, it is clear that the number of GMO-associated laboratory accidents is very scarce in comparison with non-GMO-associated infections, and practically restricted to accidental infection with recombinant vaccinia virus. We interpret this finding that the biological containment obtained by attenuating GMOs is possibly a major factor in preventing their transmission. However, other factors contributing to the low number of GMO-associated accidents may be that GMOs are well characterized, implying that the worker has knowledge of the properties of the GMO, and that the work load involving GMOs is likely much lower compared to that in clinical laboratories. Moreover clinical samples may contain unknown pathogens. Other factors that may contribute to this low number of accidents involving GMOs, are the stricter regulatory framework and a stricter compliance to containment rules. In many countries, incl. the Netherlands, both the researcher and the regulator make a risk assessment for each individual project that involves GMOs, a practice that is less developed for work with non-GMOs. In case of doubt or uncertainties regarding the properties of GMOs, biosafety experts and regulators will demand a higher risk category or additional measures. Both a local biosafety officer and a national inspectorate supervise this practice. Whenever possible we consider it important to further optimize the possibilities of employing genetic modification to enhance the safety of GMOs. In particular we recommend to further define the genetic properties underlying transmissibility and infectivity of microorganisms, and to measure the influence of specific mutations on infective dose and transmission properties of GMOs, and to base containment rules on such findings. This is not an easy task, but would further provide a scientific basis on phenotypic properties of GMOs and the accompanying level of biological containment afforded by specific genetic alterations. Vaccinia virus or recombinant viruses developed from nonhighly attenuated vaccinia viruses appear less suited as vector organisms due to their retained virulence and low infectious dose, and should be replaced by safer poxvirus vectors wherever possible.

In many reports of LAIs there has been a non-compliance with biosafety practices. This observation may be reassuring regarding the effectiveness of such biosafety practices at least if they are followed. It illustrates that education of laboratory personnel and compliance with the rules remains top priority. Increased attention to these aspects may have caused a decrease in the rate of LAIs per person per year (Osborne et al 1999, Wilson and Chosewood 2007). On the other hand, in the majority of cases of LAIs a direct cause could not be assigned (Harding and Byers 2006, Pike 1979, Sewell 1995, Wedum 1961, Yagupksy et al 2000), suggesting that a failure was not noticed in many cases, or that containment may have been

insufficient. This observation may warrant further research of the routes of exposure in such cases and the effectiveness of measures. Finally, although monitoring of LAIs is an important element of evaluating the effectiveness of containment measures, it may overlook the risks associated with non-replicating agents, such as transduction by non-replicating viruses.

Many countries, incl. The Netherlands, differently regulate work with pathogenic microorganisms and GMOs. Because the regulations are derived from the same underlying principles and use the same instruments for biosafety, and because the number of accidents involving GMOs is very low, we recommend to harmonize, modernize and simplify the regulatory framework through developing a single set of regulations for both non-GMOs and GMOs.

Despite their presumed overall effectiveness in providing biosafety, it is often unclear to what extent the current set of specific biological of physical containment measures, alone or together, contributes to the prevention of transmission of pathogenic microorganisms or GMOs. In further developing and modernizing the biosafety practice, we therefore recommend developing evidence-based practices and criteria to evaluate effectiveness wherever possible and feasible. This may optimize and perhaps simplify future biosafety measures and stimulate compliance with the rules. Although scientific research may strengthen the evidence base of biosafety measures, such work is complicated and does not necessarily guarantee new findings on which further improvements can be based. To unravel complexities and to obtain further insight in the contribution of specific elements to biosafety, mathematical modeling, which is directed on quantitative parameters of infectivity and transmission, may be supportive, but modeling evidently needs confirmation by observational and experimental findings. Such an approach may however point to the data that are needed to further guide the development of evidence-based risk analysis and containment policy for both non-GMO pathogens and GMOs.

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Annex

Table 11. Causes and factors involved in laboratory-acquired infections

Microorganism	No. of cases	Setting	Possible primary cause	Other factors associated	References
GMOs					
Recombinant vaccinia virus	1	research lab	Unknown. No apparent failures in handling; no disruptions of epidermal barrier	Possibly enhanced infectivity due to immunomodulating insert (LFA/ICAM-1); long interval after vaccination	Mempel et al 2003
Recombinant vaccinia virus	1	research lab	Needle stick injuries		Openshaw et al 1991
Recombinant vaccinia virus	1	research lab		Infrequent eye protection, laboratory coat sleeves were not elasticized and did not always cover the wrist, waste pipettes were not disinfected before removal from the biosafety cabinet, work (including vortexing) outside the biosafety cabinet, no vaccination	Lewis et al 2006

Non GMOs					
Vaccinia virus	1	research lab	Accidental cut wound on a cover slip	No vaccination	Wlodaver et al 2004.
Vaccinia virus	1	Research lab	Accidental needle stick injury	No re-vaccination	Moussatché et al 2003
SARS-CoV	1	research lab	Cross-contaminated West Nile virus sample, but no clear recognized laboratory accident.	Insufficient training and non-compliance with BSL-3 procedures	Lim et al 2004
SARS-CoV	1 (90 persons quarantined)	research lab	Spilling accident (in BSL-4 laboratory)		Normile 2004
SARS-CoV	2 and 8 secondary cases outside the lab, of which 1 was lethal	research lab	Inadequate inactivation of SARS virus batch followed by transport to low-safety lab	Non-compliance with procedures (no check on inactivation, no monitoring of workers' health status). In addition there were problems with improper air circulation, poorly located autoclaves and freezers, and wanting training	Enserink and Du 2004

				and record-keeping (Normile 2003).	
SARS-Cov	2	research lab	Unknown; perhaps inadequate inactivation and infection outside the BSL-3 area		Normile 2004
Marburg virus	25 and 6 secondary cases among medical staff (7 deaths in total)	research lab	Handling Vervet monkeys originating from Uganda before the virus was designated a risk-4 organism.		Smith et al 1967, Feldmann et al 1969, Swanepoel 2001
Marburg virus	2 (Marburg, Germany, and Belgrade)	research lab	Accidental infection before the virus was designated a risk 4 organism		Nikiforov et al 1994, Borchert 2001
Ebola virus	1		Accidental needle inoculation while processing material from patients in Africa		Edmond et al 1977

Ebola virus	5 people exposed, but not ill, likely because the strains used were not very virulent or because of infinitesimal doses (Reston, Virginia, and Fort Detrick, Frederick, Maryland, USA)		Two accidents, while handling non-human primates and mice		(ProMED-mail, EBOLA VIRUS, LABORATORY ACCIDENT - USA (MARYLAND), ProMED-mail 2004; 2 February: 20040220.0550. <http://www.promedmail.org>. Accessed 22 September 2007).
Ebola virus	1 (lethal) (Novosibirsk, Siberia)		Needle stick injury while handling guinea pigs		ProMED-mail, Ebola, lab accident death - Russia (Siberia), ProMED-mail 2004; 22 May: 20040522.1377.

					<http://www.promedmail.org>. Accessed 22 September 2007
Lassa virus	1		Handling tissue cultures and infected mice		Leifer et al 1970
Sabiá virus	2 (Brazil and Yale University)	Diagnostic and research laboratories	Handling of unknown virus (in diagnostic laboratory) and leakage from centrifuging tubes (in BSL-3 laboratory)		Barry et al 1997, Gandsman et al 1997, Lisieux et al 1994
Burkholderia mallei	1	research lab	Non-compliance with biosafety practices (no routine use of latex gloves)	Researcher had type 1 diabetes mellitus	Srinivasan et al 2001
E. coli O157:H7	5	different diagnostic labs	Not identified or not reported	Infections occurred before *E. coli* O157:H7 was reclassified as a risk-goup 3 organism	Coia 1998
E. coli O157:H7	4	different diagnostic labs	Non-compliance with biosafety practices (handling without latex	Sudden increase in volume of specimens, low infectious dose, prolonged survival on	Spina et al 2005

			gloves, hands were not washed each time that gloves were removed, open laboratory coat)	stainless-steel surfaces (Willshaw et al 1994)	
Brucella melitensis	5	diagnostic lab	Not identified or not reported		Gruner et al 1994
Brucella melitensis	7	diagnostic lab	No apparent failure of recommended safety practices	Large number of isolates of *Brucella* spp. handled per year in endemic area	Yagupsky et al 2000
Brucella melitensis/abortus	75 (retrospective survey of 30 years; attack rate 11.9 %)	different diagnostic labs	Break in biosafety measures in 80 % of the cases (including lack of recognition of an isolate of *Brucella* spp. and failure to work in a biological safety cabinet)	Large number of isolates of *Brucella* spp. handled per year.	Bouza et al 2005
Mycobacterium tuberculosis	7 (retrospective survey)	different public health labs	Needle stick injury (one case). In the other cases, the source of infection could not be determined.	Inadequate isolation procedures, high volume of specimens, faulty ventilation	Kao et al 1997
Neisseria	16	different	No respiratory protection		Sejvar et al 2005.

meningitidis	(retrospective survey)	research labs	in 15/16 cases		
Neisseria meningitidis	2	two clinical labs		High volume of specimens and formation of microaerosols	Guibourdenche et al (1994).
Neisseria meningitidis	5 (retrospective survey; relative risk for laboratory workers 184; 95 % CI 60 – 431)	different labs	Working outside a biosafety cabinet		Boutet et al 2001