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EURO-CARES

A PLAN FOR EUROPEAN CURATION OF RETURNED EXTRATERRESTRIAL SAMPLES

WP2 - Planetary protection
Deliverable 2.2- Biohazard and Biosecurity

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1. Introduction

The return of extra-terrestrial samples to Earth will be regulated under planetary protection guidelines. More specifically the handling and investigation of returned samples will need to satisfy category V of the planetary guidelines [1]. Planetary protection guidelines have previously been discussed in more detail in the first deliverable of the Planetary Protection work package – Review and Current State-of-Art (INAF-PP-REQ-WP2-TN001). Briefly, category V is separated into unrestricted and restricted sections. Unrestricted missions will return samples from locations judged by scientific opinion to have no indigenous lifeforms, so there are no special requirements to protect the Earth's biosphere, for example samples returned from the moon. Restricted missions will return samples from solar system bodies that potentially contain life, for example Mars. Unsterilised samples and any hardware that directly contacted the target body of samples from that body must remain in containment until sterilised or no life is found in the samples [1].

The facility that will receive any restricted missions must be able to therefore hold the samples within containment that will stop any release of an unsterilised particle. Specifically the Planetary Protection requirements states that the probability of a single unsterilised particle of ≥0.2 µm being released from this facility shall be ≤ 1×10^{-6} [2]. The highest level of containment currently used for biological materials is the Biosafety Level 4 (BSL4) laboratory. These are designed to allow handling and safe storage of the world's most pathogenic agents. The laboratories are designed to meet requirements defined by international and national bodies [3], ensuring biocontainment through a number of measures, such as, but not limited to, primary and secondary containment, staff selection and safe working practices. The measures already employed for high containment facilities demonstrate that the majority of the technology for a restricted earth return mission is already present, these can be built upon with technologies adapted from the pharmaceutical industry and developing technologies such as the double walled isolator and robotic manipulation.

This report will touch upon the requirements for other work packages that relate to this topic but will focus on the biohazard and security aspects of a facility relating to planetary protection for restricted Earth return samples. As unrestricted Earth return missions will not require containment to protect the Earth's biosphere then they will not be considered within this report, apart from the use of containment systems to protect them from Earth contamination. This will also be a focus of restricted Earth return missions where the sample will need to be contained from the Earth biosphere to avoid contamination but also to ensure the Earth's biosphere is protected from the sample.

2. Hazardous agents

There is international and national guidance on the description and classification of a Risk Group (RG) 4 agent which might vary slightly between guidance and legislative documents. In the UK biological agents are classified into different risk group based on evidence of the agents:

- Likelihood to cause disease by infection or toxicity in humans
- Ability that the infection would spread to the wider community
- Is treatable by prevention (prophylaxis) or intervention after infection [4].

In Europe it is organised through the Directive 2000/54. However there is not process to update this list as agents emerge or become better understood.

A further definition is given by the World Health Organisation (WHO), describing a RG4 agent as:
"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 preventative measures are not usually available [5]."

At present all RG4 agents are viruses, including agents such as Ebola, Marburg, Lassa and Crimean-Congo Haemorrhagic Fever. Viruses are obligate intracellular pathogens and are incapable of replication outside the host organism except in host derived cell lines used in laboratories. There are thought to be millions of different viruses, which are able to infect both Eukaryotic and Prokaryotic cells. Whilst these RG4 agents are extremely infectious and have high mortality rates they only survive outside of their host for short periods of time. This also means that their decontamination can be easily achieved, but might become more difficult if associated with organic material [6]. It is extremely unlikely that a virus returned in a n extra-terrestrial sample will pose a hazard to terrestrial life forms as viruses co-evolve with their host. Viruses are usually specific for a certain cell type or species of organism, therefore a virus that can infect one organism probably will not be able to infect an organism of a different species.

The environmental conditions that life would be exposed to on a body where life detection or return mission searching for life would be targeting, such as Mars, would select for the comparatively hardier organisms on Earth, rather than less resistant viruses. On Mars there are a number of different environmental factors to which any organism would have to have adapted to survive. The factors are the reduced atmospheric pressure, a large fluctuation in the temperature (especially freeze-thaw), reduced moisture (desiccation) and an increase in exposure to galactic cosmic radiation. A low available oxygen content of the air would favour capnophilic organisms which can grow and replicate in atmospheres of high CO_2 using fermentation as their means of producing energy. There are a number of commonly found capnophilic organisms found on Earth e.g. *Clostridium difficile* [7]. The response of terrestrial microorganisms has been investigated against the Martian environment and the radiation that they would be exposed to. Indeed, tests performed on soil samples using simulated Martian

environmental conditions showed that Earth microorganisms were able to survive under these conditions and even replicate at a slow rate [8]. Martian regolith can also help to reduce the level of radiation a microorganism would be exposed to the deeper it was. Spores exposed to UV radiation similar to that found on Mars survived for significant lengths of time when covered by only 1mm of Martian simulant regolith [9]. Therefore life could be treated as similar to the extremophiles that are found on Earth but this assumes that the life being looked for in an extra-terrestrial sample is similar to life that can be detected already on Earth. This means that essential components of an Earth organism such as, proteins, nucleic acids, metabolism by products, etc., will be similar in the extra-terrestrial life. Due to the unknown nature of any life found in a sample returned from another celestial body then it is important to use the definitions of life previously identified in Earth organisms and the prerequisites needed for life; water, carbon and energy [10].

The potential pathogenicity of any life form returned from an extra-terrestrial body to Earth is unknown but is likely to be very low. It would have to be assumed that to infect a living species on Earth then the lifeform would need to have similar biochemistry of a pathogenic organism on Earth [10]. Due to the hostility of the environments on other celestial bodies then the lifeform would need to be hardy enough to survive and replicate there, similar to the extremophiles on Earth. Conversely, the lifeform's ability to thrive in the extreme environment could stop it from becoming pathogenic. For example an extremophilic organism that is able to replicate at sub-zero temperatures might be killed at temperatures found within the human body [11], therefore decreasing the risk from infection from a lifeform returned.

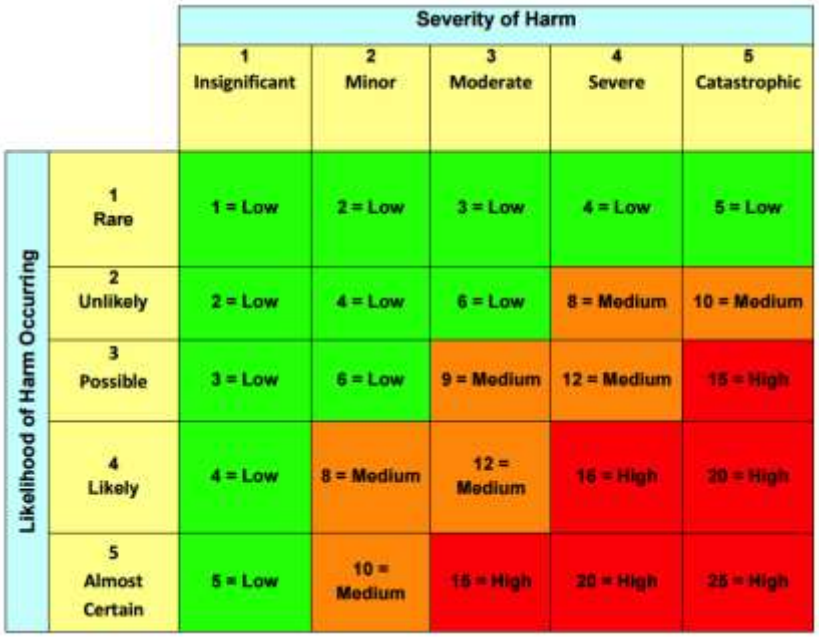
Overall, pathogenic agents on Earth have evolved with their host species to allow for their infection and proliferation within the host species. Most pathogenic species will also have an animal reservoir, which will not be found on the celestial body. Whilst extra-terrestrial lifeforms might be able to survive in extreme conditions found on those bodies they would be extremely unlikely to be able to infect and cause disease in any Earth lifeform due to the different growth conditions and not having contacted or evolved with the host target.

3. Assessment and perception of risk

Biological high containment facilities are built to prevent the transmission of the pathogenic agent to the laboratory workers and release of the agent to the outside environment. It has been accepted for many years threat any samples returned from Mars must be held in a facility with the same containment standards as BSL4 [10]. Therefore the returned material will be treated as if there is a highly pathogenic agent within it until it is proven that no extant life is present. In order to protect the workers and environment it is important to understand the risk posed by the agent(s), the activities performed, and severity if there is a release from the facility. Protection measures can be identified and put into place when these risks are identified. In terms of the return of extra-terrestrial samples, risk assessments have already be completed to assess the possibility of life from different celestial bodies. This can be seen from the planetary protection guidelines for category V earth return missions where certain bodies are deemed to have no indigenous life on them then they can be treated as unrestricted and restricted Earth return missions from bodies where there is the potential for life to be found [1].

There are a number of different methods that can be used to perform a risk assessment for a sample return mission. These allow for either a qualitative or quantitative assessment of the risk and the hazard posed. Some of the risk assessment tools are Hazard and operability study (HAZOP), Structured what-if technique (SWIFT) and Layers of protection analysis (LOPA) [12, 13]. These risk assessment tools will provide a structured approach to assessing the risk and developing action that can be implemented if an adverse event happens. Risks can quantified using two components, severity and likelihood, that can be multiplied together to give a risk rating. For instance both severity and likelihood will be scored from 1 – 5, 1 being the lowest and 5 the highest, Figure 1, and then multiplied together to form the final risk rating. The risk rating can then be determined either as low, medium or high. If risks are deemed to high then controls can be implemented to reduce them to an acceptable risk level. In certain situations it may be difficult to reduce the risk to a low level and the risk assessment will explain why a medium rating is acceptable.

Figure 1. Risk rating matrix, calculated using severity vs likelihood (Courtesy: Public Health England)



Likelihood of Harm Occurring	Severity of Harm: 1 Insignificant	2 Minor	3 Moderate	4 Severe	5 Catastrophic
1 Rare	1 = Low	2 = Low	3 = Low	4 = Low	5 = Low
2 Unlikely	2 = Low	4 = Low	6 = Low	8 = Medium	10 = Medium
3 Possible	3 = Low	6 = Low	9 = Medium	12 = Medium	15 = High
4 Likely	4 = Low	8 = Medium	12 = Medium	16 = High	20 = High
5 Almost Certain	5 = Low	10 = Medium	15 = High	20 = High	25 = High

Biological risk assessments are used to help drive the level of biosafety containment and handling practices of the samples. When completing the risk assessment a number of different factors should be included:

- Agent pathogenicity
- Severity of exposure
- Route of infection of the agent
- Volume and titre of the agent handled
- Persistence of the agent in the environment
- Environmental impact, susceptible species and availability of vectors

All of these are unknown for any agent in a Mars sample.

Risks will vary depending on the type of sample that is collected and/or the type of analysis that is being undertaken. It is envisaged the majority of the samples return from extra-terrestrial bodies will be rock cores, regolith material and some atmospheric gases. If agents are present in these samples then the most likely infection routes would be either inhalational (small particulates aerosolised) of through direct contact (surface contamination). Current working protocols, employee training and engineering controls in high containment laboratories are designed to reduce the likelihood of release and/or infection through these routes using primary containment and PPE. It is difficult to estimate the level of hazard from the release of an extra-terrestrial lifeform due to the uncertainty about the pathogenicity or toxicity to the environment. Therefore it should be treated at the highest level of hazard. This will therefore potentially increase the risk rating into the medium or high risk areas and the identification that control measures should be used to reduce the likelihood of a release of an unsterilised particle.

There is also the added complexity of public perception of the risks that are involved with the facility. Whilst efforts will be made to ease the public's fears through outreach and education there will always be a level of scepticism about intentionally returning samples from another celestial body in an attempt to search for life and that this potential life might escape to Earth. Public perception has also been directed towards BSL4 facilities where perception of the agents handled in these facilities has been coloured by fictional representation, in some ways to a lesser degree than alien life forms have been in the Sci-Fi genre [10]. Microorganisms fulfil many of the features of the fear factors defined by Bennett *et al* in the book Risk Communication and Public Health [14]. With factors more worrying if perceived as; to be involuntary rather than voluntary, to arise from an unfamiliar or novel source, to cause hidden and irreversible damage, and to be poorly understood by science. But as public confidence has grown in the biocontainment of the facilities they have started to be accepted more. This might be the feeling towards a sample receiving facility where there would be a mix of fascination and trepidation towards it.

Overall the use of a risk assessment can identify areas or tasks that will require interventions to reduce the risks associated. Whilst there are a number of unknowns when handling restricted Earth return samples in a facility, such as type and concentration of any agent within the sample, engineering and protocols can be put into place to mitigate the risks. The assessments can be generated using worst case scenarios with Earth organisms to provide a more quantitative approach. Assessments can be further modified using knowledge gained from the sample collection missions where it would be assumed that life detection analysis of the environment would be undertaken and the information used to update the scientists dealing with the material on its return.

4. Documentation and regulations on the containment of biohazardous agents

Governments and international organisations have published legislation or guidance on the containment of biological agents. The World Health Organisation (WHO) has produced the Laboratory Biosafety Manual [5] and the European Union (EU) that has produced the Directive 2000/54/EC [3] regarding biological agents at work and the design and operation of a biological laboratory.

Some countries' regulatory bodies have produced their own biosafety guidance documents that can be used as the principles for the design and operation of a BSL4 facility. The UK's Health and Safety Executive (HSE) has produced Biological agents: The principles, design and operation of Containment Level 4 facilities [4]. This guidance document details the legal requirements of working with high hazard pathogens within the UK. The American Centre for Disease Control (CDC) has produced the Biosafety in Microbiological and Biomedical laboratories [15], which is an advisory document for the best practices of conducting work in the laboratory from a biosafety perspective. The Russian government have produced a number of State Sanitary Rules that relate to the safety of working with microorganisms and the organisation of high containment microbiological laboratories [16].

As an addition of these guidance documents there are further legislations that need to be adhered to if certain biological agents are handled in the facility. The biological agents (bacteria, viruses and toxins) that have been decreed to have a potential to cause severe public health and safety threats and have greater restriction on their use. This is to stop any misuse from these agents from deliberate release. Further inspections are necessary by the regulatory body to ensure appropriate precautions for biosafety and biosecurity are in place within the facility. The WHO has published guidance on Biorisk management focussing on laboratory biosecurity guidance [17]. Within the UK this is applied by the Anti Terrorism Crime and Security Act (2001) and the biological agents covered by this are listed in Schedule 5 of the act. Within the US a similar law has been passed since 1997 entitled Biological Select Agents and Toxins. The International Air Transport Association (www.iata.org) have published a regulation document on the transport of dangerous goods [18]. It requires the sample to be packaged in accordance with WHO packaging guidelines [19].

There are also biosafety documents, guidance and training provided by non-profit organisations, in North America there is the American Biological Safety Association (www.absa.org) and in Europe the European Biosafety Association (www.ebsaweb.eu). These associations also provide networks for people to converse through and discuss biosafety issues relating to aspects of the facility.

The sample receiving and curation facility would need to be designed with the most appropriate guidance document that is relevant for the country, i.e. if there is a specific national document it will need to be adhered to, but if there is not then an international document such as the WHO's Laboratory Biosafety Manual will be used. The facility will be checked by the hosting country's regulating agency to ensure it has been designed and constructed to the regulations that are applicable.

Furthermore, it would be envisaged that during the design of such a facility a number of biosafety experts from around the world would be consulted on the design and operation of the facility.

5. Principles of high containment

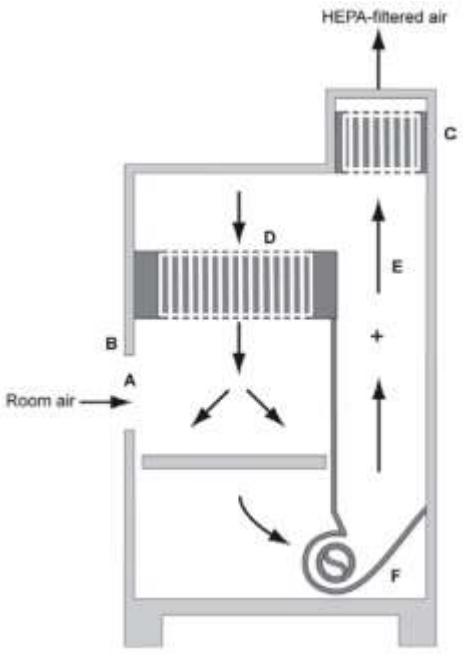
A BSL4 laboratory will consist of a number of different containment systems that will be used together to contain the agent and protect the worker and the environment. The containment can be defined as primary and secondary containment. Primary and secondary containment of a BSL4 facility incorporate engineering designs such as; biological safety cabinets, directional air flow and air handling units, the laboratory shell, personal protective equipment and effluent treatment systems. This section will discuss the different types of containment and how they may be used in a sample return facility.

Primary containment

Primary containment is designed to contain the pathogenic agent being worked with and protect the workers in the immediate laboratory environment. Primary containment uses a barrier to protect the worker from the hazard, whether it is a physical barrier, the use of directional airflows or a combination of both. Examples of these are microbiological safety cabinets (MSC) and sealed centrifuge rotors.

The two different types of laboratories (suited and cabinet line, Figures 3 and 4) used at BSL4 will both require the use of MSCs for handling and procedures manipulating the agents within them. In suited laboratories open fronted class II MSC cabinets are used, the cabinets require operators to work in the cabinet through an air curtain with air drawn in from the surrounding laboratory and passed through a HEPA filter before entering the working zone, Figure 2.

Figure 2. A Class II, Type A1 MSC. (A) Front opening, (B) Sash, (C) Exhaust HEPA filter, (D) Supply HEPA filter, (E) Control plenum, and (F) Blower unit. The arrows indicate the direction of the air movement. Reproduced from the Biosafety in Microbiological and Biomedical Laboratories, 5th edition [15].



Some of the air is recirculated and then exhausted through double HEPA filters. Due to no physical barrier existing between the worker and the agent positive pressure suits are worn, these protect the worker from aerosol and surface contamination using filtered breathing air to positively pressurise the suit to limit the ingress of any aerosolised particles, the suit's waterproof material will stop deposition onto the worker's skin. In a suited laboratory there will be no manipulation of infectious material outside of the primary containment.

In the cabinet line laboratory all infectious material is manipulated through gauntlets within a connected chain of class III MSCs. The workers do not wear positive pressure suits because there will be no direct contact between themselves and the agent. The cabinet line is operated at a high negative pressure, with the air of the cabinet HEPA filtered on the inlet and double HEPA filtered on the extract. Class III MSCs are also known as glove boxes and are tested to be air tight if the inlet and exhaust filters are closed off. Glove boxes also will provide a greater level of protection to the sample from Earth contaminants so would be preferable for handling and analysis of unrestricted Earth return samples. Although the protection offered would only be particulate as off gassing of chemicals can still occur, whilst the high air change rate and turbulent flow would be unsuitable form handling small dusty particulate samples.

Similar to class III MSCs, flexible film isolators are used in high containment laboratories, where they are generally used for handling of infected animals. These have a rigid external metal frame that supports a flexible canopy acting as the physical barrier. Glove ports are located along the sides of the canopy, for larger isolators half suits are fixed to the base and operators crawl underneath to enter them (Figure 5). These isolators also operate at negative pressure to the laboratory usually at a reduced level to that of a class III MSCs to reduce the stress placed on the animals contained within.

Figure 3. A cabinet line laboratory

Figure 4. Operators within a suited high containment laboratory

Figure 5. A flexible film isolator used at BSL4 at PHE Porton Down

Secondary containment

Secondary containment is the use of the laboratory design, construction, engineering controls to protect the environment and other operators of the facility from infection by the pathogenic agents being handled. Examples of secondary containment are the use of ventilation to create negative pressure and inflow of air, and the sealability of the laboratory structure itself.

In high containment laboratories around the world negative pressure and inflow of air is used to prevent egress of infectious aerosols. There is no internationally agreed pressure or air change rates recommended for high containment laboratories and they vary in existing facilities. The pressure differential is a measurable indication that any aerosol leakage from the laboratory will travel towards the area that is most hazardous and away from the environment external to the facility. The UK Health and Safety Executive guidance states that for BSL4 the minimum pressure differential between the laboratory and the ambient environment must be -75 Pascals, with 30 Pascals between each layer of the facility [4], where there is no specific pressure differential required by the US BMBL guidance. The BSL4 facility in Lyon, France uses a pressure gradient of -40 Pascals at the inner changing room down to -200 in the post mortem and radioisotope rooms [20]. Whilst a large negative pressure differential is seen to assist in the containment of pathogenic agents it can add complexities to the facility and greatly increase the cost of building and running of the facility. Buildings must be built to withstand the effects of negative pressure.

Another factor in secondary containment is the air change rate within a facility. Whilst the general preconception is that a high air change rate within a laboratory is an indication of high containment performance there is little evidence as to what a BSL4 facility should achieve in normal operation. Air change rates might only be increased if they are to help deal with any heat loads or non-microbial contaminants.

Table 1. Time taken in minutes for removal of pathogen percentage in the laboratory depending on the air change rate per hour (Table is replicated from future HSE guidance on the management and operation of microbiological containment laboratories [21])

Air changes per hour	**Time taken in minutes for the removal of the percentage of aerosols**			
	90	***99***	***99.9***	***99.99***
10	14	28	41	69
20	7	14	21	35
30	5	9	14	23
40	3	7	10	17

Pressure hold testing is often used to assure that the design and construction has provided a leak tight facility. Pressure tightness is specified to ensure that there are no leaks of fumigant during the

decontamination process and to set a leak rate to define the build quality of a facility, setting the standard for subsequent maintenance.

A trade off will need to be made when designing a receiving and curation facility for the handling of both restricted Earth return samples to establish what method of containment should be used. In relation to the prevention of aerosol release to the Earth's biosphere from the laboratory the existing primary and secondary containment used in BSL4 facilities provides a high level of assurance that there is no release.

Suited systems allow greater flexibility in the handling and manipulation of sample containers, but, they would also potentially lead to contamination of the samples. The suit material itself would need to be investigated to establish whether there is off gassing. The exhaust system of current suits uses a one way valve that is not HEPA filtered, so air coming from the suit would therefore contain biological and physical contamination from the user. Conversely cabinet lines are more restrictive for the user but conditions within them can be controlled more easily and cost effectively than an entire laboratory e.g. ultrapure nitrogen atmosphere. But cabinet lines are rigid in their design. They can only be a certain width due to the restricted reach of the operator, therefore they will be restrictive in terms of the size of pieces of equipment that can be housed within them. Alternative design modifications could be used to ensure protection to the sterility of the sample and to the user and surrounding environment, these could include the use of robotic manipulators within the containment to remove any direct human interaction.

6. Other considerations for the safe working of a high containment facility and reduction in exposures to hazardous biological materials

Decontamination of the laboratory and the waste

Laboratories use a variety of decontamination methods to ensure waste liquids and solids and laboratory surfaces are sterilised. Decontamination can be used prior to commencing work to ensure the area is sterile, after work to decontaminate the area before the next sample is processed or after an incident to ensure the area is safe to return to for the workers.

Liquid waste in small volumes is normally treated with a chemical decontaminant in the cabinets within the laboratory before subsequent autoclaving. Large volumes of liquid waste, typically from washing of positive pressure suits or personal showering is pooled in a specialist effluent treatment plant and treated using either a validated chemical or a physical methods usually heat. Solid waste is usually dealt with by autoclaving [22].

Surfaces within the cabinet will be initially treated with a validated liquid decontaminant for a specified contact time. The final step in the decontamination of a high containment laboratory will be using gaseous decontamination. This requires the introduction of a gas or vapour into the chamber for a specified time to inactivate the agents. There are a number of different technologies and techniques that can be used, for example formaldehyde, hydrogen peroxide and chlorine dioxide [23]. Validation will be undertaken to ensure that they are effective at the temperature, concentration, exposure period and against the agent that is needed to be inactivated [6].

The application of disinfectants (liquid and gaseous) may cause the formation of residues on or off gassing from surfaces. Many chemical decontaminants may oxidise or damage surfaces. A study may be necessary to investigate if any material damage is seen over a number of applications. It might be important to determine if sensitive equipment will be affected by the decontamination process [24].

Planned preventative maintenance

In high containment facilities the laboratory will be fumigated and made safe for entry by engineers to service and maintain equipment on a regular schedule, normally every six months. An emergency shut down of the laboratory might be required if critical equipment fails. A high containment facility should be designed to aid the servicing and repair of the equipment by ensuring that most of the equipment will be housed outside of the containment and only necessary parts within it, for example having a Bluetooth keyboard and mouse for the computers within the laboratory and the computers and servers external to it, or designing lighting so that the bulbs can be replaced from outside of the containment envelope.

Showering

If the high containment facility uses positive pressure suits then they must be decontaminated after leaving the laboratory and prior to doffing. Currently suited laboratories will have a shower room, with sealable doors and exhaust air handling, on the exit of the laboratory. A validated chemical shower cycle is used to remove and decontaminate the suit from any RG4 pathogens than might be present on the exterior surface. As a redundancy the shower will be built with a gravity fed tank with pre mixed decontaminant that can be used if the power supply is lost to the facility. In a sample return facility it will be important to validate a chemical that is able to both wash contamination from a suit and also decontaminate the waste to a suitable level. This will be validated with Earth organisms in a stringent test to ensure it is effective and reproducible. It would also be prudent to ensure the suit would be able to maintain its integrity after multiple treatments with the decontaminant as these can be corrosive not only to the suit, but glue and zip materials.

Personal showers are used in both types of high containment laboratory (suited and cabinet line). Operators are required to shower out from the laboratory after finishing their work. Waste from the personal shower in the suited laboratory is discharged direct to drain as the suit will provide the protection to the worker. The effluent from the personal shower in the cabinet line laboratory is treated before being discharged.

Standard operating procedures

The use of a standard operating procedure (SOP) will allow all staff to be trained to the same standard, which will have been tested and validated when required. This is important for high containment laboratories where systems of safe work can be identified and then implemented. SOPs can be applied to the use of equipment within the laboratory, the entry/exit routine and emergency procedures. Training will be given on the SOPs and regular checks will be made to ensure they are current with the technology and safety practices.

SOPs for emergency measures will depend on the design of the facility. Certain aspects must be taken into account such as staff safety and sample preservation. For example is there a need for break out panels within the laboratories to allow emergency exit during fire? In this case would it be acceptable for potential release of a lifeform if the laboratory was exited with PPE removal and showering? What are the emergency measures regarding fire suppression? If a water based suppression system is used then where will the waste water collect? Will it be treated before release, which dependant on the volume, this might not be possible so contamination from the run off might occur. If an inert gas suppression system is used will there be respiratory protection for the workers and will the added

pressure of the gas turn the laboratory positive in pressure to the external areas potentially releasing aerosols? These aspects will need to be addressed during the planning phase and a strategy for the design determined.

Biosecurity

Biosecurity is the provision of interventions that will stop the deliberate removal or release of the hazardous agents from a facility. The facility should be designed to limit the access of non-critical staff to it. This will include security guards on the entrance, security fencing to maintain a secure enclosure around the facility. Within the facility itself there should be doors that are access controlled allowing only those with the right level of clearance through them. This might be through physical methods, e.g. security key cards, or using biometric control, e.g. fingerprint or retinal scan. Extra controls should be placed on any storage areas where samples will be kept because access to this area will give access to large numbers of the samples in one place.

It will be necessary to perform security checks on the individuals working in the establishment and if there are facilities or samples provided by more than one nation then these checks might be necessary to carry out from all of the supporting nations.

7. Current test methods for the detection of pathogenic organisms

A draft protocol has been published by NASA in 2002 which details the testing required to detect biohazards in Martian samples returned to Earth [25]. This protocol was developed after a series workshops and working group meetings from 2000-2001. It was entitled 'A Draft Test Protocol' because it was felt that the "process of assessing the Martian samples should owe much to new knowledge about Mars that will be gained in robotic exploration" and "detailed information available only on the sample return mission itself" this would also include the nature of the receiving facility and the detection instrumentation that is available for use. The draft protocol has been used to determine a "sufficient approach" for the life detection of Martian samples returned to Earth using a step wise process [25]. The technology used within science is rapidly advancing since this protocol was written in 2002.

There are a number of chemical, physical and microbiological tests that can be undertaken to determine if life or specific markers for life are present in a sample. The initial testing campaign proposed by Rummel *et al* suggests a step wise approach attempting to answer 4 questions using different technologies and methodologies for life detection which is shown in Table 2 [25].

Table 2. Step wise approach for investigating for life in returned samples [25].

Item	Question	Strategy
1	Is there anything that looks like a life-form?	Microscopy; beam synchrotron or other non-destructive high-resolution analytic probe, particularly one that would allow testing unsterilized (yet still contained) samples outside main facility.
2	Is there a chemical signature of life?	Mass spectrometer and/or other analytical measurement systems (to be used in containment) that would identify biomolecules, chiral asymmetry, special bonding, etc.
3	Is there any evidence of self-replication or replication in terrestrial living organism?	Attempts to grow in culture, in cell culture, or in defined living organisms.
4	Is there any adverse effect on workers or the surrounding environment?	Microcosm tests; medical surveillance of workers and monitoring and evaluation of living systems in proximity of receiving facility to ensure no release or exposure associated with operations of SRF.

A more recent workshop was held in 2014 that proposed more updated methodology for the life detection in Martian samples returned to Earth. This workshop updated elements of the Planetary Protection Draft Test Protocol and uses the definition for signs of life to be "For the purposes of the Planetary Protection Test Protocol, any indications of viable, dormant, or recently deceased life forms, as well as fossils and the trace evidence of life processes" [26]. From this workshop an updated sequence of analysis for the samples returned was proposed and is shown in Table 3 below.

Table 3. Sequence of analysis to be performed on Mars at the time of sampling and when returned to Earth [26]

Sequence for sample analysis	Sample condition	General type of analysis
I	Sample acquisition on Mars	Remote and *in-situ* analysis on Mars to characterise the sample type and the geological context
II	Any solid sample material on the outside of the sample containers	Solid sample analysis; full sequence (non-destructive & non-invasive, non-destructive & minimal invasive, and destructive)
III	Head space gas	Gas sample analysis; full sequence
IV	Solid samples in containers	Solid sample analysis; non-destructive & non-invasive
V	Solid samples removed from containers	Solid sample analysis; non-destructive & minimal invasive
VI	Fluid inclusions from solid samples removed from containers	Liquid sample analysis; full sequence
VII	Solid sample removed from containers	Solid sample analysis; non-destructive & minimal invasive, destructive

The techniques to be used in the detection of evidence of life were also characterised depending on their destructiveness and their use of different sample types. These are described in Table 4

Table 4. Example sample analysis techniques that can be used for life-detection [26]

Invasiveness	Solid sample analysis	Gas sample analysis	Liquid sample analysis
Non-destructive & non-invasive	• 3D X-ray micro-tomography • Surface imaging and spectroscopy	Not applicable	Not applicable
Non-destructive & minimal invasive (no specific sample preparation)	• Microscopy • Fluorescence • IR, visible, UV, deep UV spectroscopy • SEM	• IR, visible, UV, deep UV spectroscopy	• Microscopy • Fluorescence • IR, visible, UV, deep UV spectroscopy
Destructive (specific sample preparation)	• SEM, TEM, nano-X-ray-tomography • XRD, XANES • GC-MS, GC-IRMS, FTICR-MS, LC-MS, TOF-SIMS, Nano-SIMS • Target independent biopolymer sequencing	• GC-MS, GC-IRMS, FTICR-MS, LC-MS	• GC-MS, GC-IRMS, FTICR-MS, LC-MS, TOF-SIMS, Nano-SIMS, • Target independent biopolymer sequencing, flow cytometry

These identified techniques will be able to detect the molecular markers for life but does not address the use of conventional microbiological techniques as are addressed in Planetary Protection Draft Test Protocol [25]. Within the protocol it is proposed that culture based methods should be used in order to rule out any contamination of samples with Earth based organisms and also to attempt to culture organisms under Mars-like conditions. This will include biohazard testing where samples from the returned mission will be taken and used to determine if they are pathogenic to organisms and vegetation on Earth.

Conventional microbiological life detection tests can be split into molecular techniques, culture based and animal infection model methods. Brief descriptions of the testing procedures are given below.

Molecular techniques

These detection techniques will investigate the presence of a target organism in a sample by looking for part of that lifeform. These techniques determine whether the test target is in the sample but will not be able to determine if the lifeform is viable or non-viable. For example polymerase chain reaction (PCR) can be used to amplify a specific DNA sequence of a bacteria's genome. The genome will be held

inside a viable bacterium where the cell will be intact. During the processing step the cell will be broken open to expose the DNA and allow for the amplification to then be detected. But if a cell has been damaged, and non-viable, yet still contains the DNA or destroyed completely and the DNA is free in the sample then PCR will still amplify that sequence if it is present. Therefore it is difficult to accurately determine if a sample contains viable or non-viable microorganisms. Other methodologies for PCR use 'universal primers' that anneal to conserved regions, in particular within the 16s ribosome region. But these regions will only be shared across species or organisms, so again specificity of the sequence is required [27]. The restriction of PCR is that it relies on prior information for the design of primers to amplify the target sequence up which with a truly unknown organism will involve guesswork.

Other molecular genetic techniques can be used to detect the presence of a microorganism or part of that microorganism within a sample, by detecting antibody reactions to specific antigens. Enzyme-Linked Immunosorbent Assays (ELISA) use a biochemical assay to detect the presence of an antigen in a sample. The sample containing the antigen will be added to a microtiter plate, where binding of the antigen occurs to the plate's wells through charge interaction. Then a primary antibody (with conjugated enzyme) is added which binds to the antigen. After which a substrate molecule for the enzyme is added which in the presence of the enzyme will be catalysed and produces a colour change which is measured through a plate reader. After validation the stronger the colour change represents a higher concentration of the original antigen in the sample. This method is usually used to detect antigens but can be used with whole bacterial cells and viruses.

Whilst both of the mentioned techniques can have high specificity and detect low concentrations of agents, but both rely on previous knowledge of the microorganism where at least part of the DNA sequence is known, or the antigen is known (such as a toxin or protein). Next generation sequencing can be used to detect a number of organisms within a sample. This approach identifies the organisms' genetic signatures within the sample. The sequences produced can then be assembled using a computer and compared to a database of sequences. This will identify if an entire sequence or specific section has previously been identified [28]. In a mixed organism sample if sequences are known they can be allocated to existing organisms and any remaining signatures will relate to unknown organisms [29]. One of the main issues is the analysis of data, vast numbers of small sequences will be produced from one samples. Powerful computers are required to analyse these data and match the sequences together [30].

Culture techniques

Traditional culture techniques require the addition of a viable biological agent to growth medium or living tissue that it can replicate in followed by incubation at the correct temperature, time and conditions (e.g. atmospheric composition). The growth of the agent can then be established by counting the colonies form on a solid media plate, the turbidity of a liquid growth media or the zones of clearing through cytopathic effects of viruses. Culture of an agent will allow the worker to determine if

it is viable in the majority of cases. There are exceptions to this which could lead to false negative reports when no growth or replication is seen. It is necessary to establish the growth media or tissue for the agent because without this then the agent will not be detectable. Examples of this are:

Viruses will grow in specific cell cultures, Influenza virus is primarily grown in Madin-Darby canine kidney epithelial cells but will not grow in a number of other cell types. This means that to identify a virus that is not characterised will mean using a number of different cell lines to establish growth.

The physical growth conditions will also be pivotal in the growth of an agent. Organisms, particularly human pathogens will have adapted to grow at a temperature around that found in the human body 32-37^{o}C. Therefore when grown they will tend to replicate best at this temperature range. Again for an uncharacterised environmental organism a range of temperatures will be necessary to establish growth potential.

The time required for an organism to replicate will be a function of that organism and the environment it persists in. In some environments, such as sub surfaces below the oceanic floor, prokaryotes have been postulated to have a turnover (doubling) time of 1-2 10^3 years [31]. The detection of organisms that are slow growing might not be reliant on the formation of colonies on or in media. Mycobacterium tuberculosis can be detected by incubating samples on selective media and using fluorescence sensors the oxygen content can be measured. The level of oxygen in the tube will be proportional to the detected fluorescence and the number of bacteria present. This system is called the Mycobacterial Growth Indicator Tube (MGIT) [32].

Growth on different media can lead to different morphologies of the colonies formed, which in turn can lead to the misidentification of the agent concerned.

The use of culture methods to grow organisms from extra-terrestrial samples will lead to issues in planetary protection, as if successful the life form will be greatly amplified. It will be necessary to identify how any growth should be dealt with and protocols developed to ensure that any life is not released from the facility. Things that will require identification are the engineering controls in place and the decontamination techniques to stop the release of the lifeform. For example will the lifeform be captured within the HEPA filters used on the containment's extract and is a combination of heat and chemicals appropriate to ensure inactivation of the lifeform in the effluent? Again the use of hardy Earth organisms will allow for the conservative validation of the engineering and decontamination approaches.

Similarly it will be a requirement for any technology that will come into contact unsterilised sample in the use of detecting evidence of life will need to remain in containment before it can be proved to be decontaminated effectively. Samples undergoing testing with potentially contaminate the machinery and therefore leave the technology contaminated after use. There will also be the requirement for testing to determine if there is any carry over contamination between samples. This can easily be

completed using negative controls between samples, but if contamination is found from sample to sample then cleaning and decontamination steps need to be identified and implemented.

If a lifeform had an extended growth period then careful interpretation of multiple test results would need to be considered to ensure that culture tests would not give a false negative result when biochemical analysis had potentially given positive results and samples treated as having destroyed or non-viable lifeforms within them.

8. Health surveillance and staff selection

Health surveillance, a system of ongoing worker health checks, will help to identify any detrimental health effects that might be caused by working with certain substances. An example of this would be the use of formaldehyde, a chemical used in the gaseous decontamination of microorganisms, would require staff to undergo regular lung function tests to ensure no ill effects are caused by its contact. Health surveillance can also be used to identify if a particular worker or group of workers might be at greater risk from certain hazards. For instance, workers undergoing antibiotic treatment are at greater risk of becoming infected by *Clostridium difficile* and therefore should limit or exclude any contact with the agent whilst undergoing treatment. Health checks for staff working at high containment facilities around the world differ depending on the country. For instance within the UK only lung function checks are required if the worker will come into contact with inhalational sensitising agent, whereas in Canada a wide variety of checks are required including but not limited to eyesight, blood tests, blood pressure, x-rays and hearing. This variance might be seen because of the difference in working practices where the UK works in physically less demanding cabinet lines but Canada uses positive pressure suits for the lab work.

Within these high containment facilities it would be prerequisite that the worker will be made aware of what symptoms of infection with the agents they are handling will be so they can contact the appropriate person if an infection is suspected. In a sample receiving facility where restricted Earth return samples are handled there should be a similar surveillance policy where staff are required to inform their management if any deviation in their health is experienced. As the samples returned will potentially have uncharacterised life in them, even at a very low probability, then the effects of the organism(s) will not be known on humans and it will be impossible to say what symptoms would be caused by an infection. One method of health surveillance that has been used in the ongoing Ebola virus outbreak of 2014 was temperature monitoring of staff members that were working at treatment centres. Workers on returning to the UK were asked to monitor their body's temperature morning and evening, if an elevated temperature was measured then they would contact the duty doctor for advice. This methodology could be used in conjunction with blood banking. This is where blood from the workers is taken before work starts and at regular intervals to allow for the analysis and comparison of the samples to establish if there is a change in the immune response, or other biomarkers, that might indicate an infection.

If a break in the containment of the facility is detected then those workers potentially exposed to the returned material may well be quarantined and observed for a period of time to assess if they had any reaction that could have been caused by an agent. This could involve monitoring of staff at set points of the day to identify changes that could be caused by an agent (temperature, blood pressure, blood oxygen etc.). As any infection would be novel to humans treatment would be of the disease manifestations.

Staff recruitment for the facility will be from those who have operated in similar working environments before. For work with high containment facilities prior work at a lower containment level is normally a

prerequisite for selection. This selects for workers that have demonstrated the necessary transferable skills suitable for the environment and an awareness of the risks involved when handling live samples. Further training is then given to workers before they are allowed to work on the live agents. A number of procedures must be observed first, then demonstrated whilst under instruction before the worker is deemed competent enough to work unsupervised. In the case of a sample return and curation facility it would advisable to have a mock up laboratory that would allow workers to be trained and existing workers to practice procedures. This area would provide necessary training for the workers to increase competency and therefore reduce any potential risks associated with working on samples in the facility and decreasing the potential of release of any agent to the external environment.

9. Conclusions

Risk Group 4 agents have been handled in BSL4 facilities since their original classification in the 1960s. Throughout this period there have only been a small number of laboratory incidents potentially or actually leading to infections in workers [4]. Some of these incidents were when the infectious agent was not known in an animal model and lead to the discovery of RG4 the agent. After this there has been a history of safe use of the agents in BSL4 facilities. It can be said that the facilities themselves have been operated and maintain correctly, because the lab incidents that have occurred have been due to operator error as opposed to equipment failure.

This highlights that when a biohazard has been identified and correctly assessed appropriate measures can be implemented to ensure that the risk from the hazard is reduced to a safe level. For instance before Marburg virus was identified in Grivet monkeys there were a number of deaths from lab workers handling them outside containment [33], after it was identified BSL4 procedures and mechanisms were put into place to reduce the risk, which eliminated the infections. This shows that the identification of biohazards from returning samples is critical in the identification of measures to maintain the safety of the workers and environment. For a sample return facility the hazards and risks will be identified before the planning stage to allow for the design of engineering features that will reduce the risk.

There are a number of different interconnected elements that are necessary for the safe functioning of high containment laboratories for Mars sample return. It will be necessary to review and analyse the tasks which will be undertaken, the type of samples that will be used and the potential for the release of an agent from the facility, these will help to develop the risk assessment for the facility and ultimately feed into its the design and construction to allow for safe operation.

As described above the facility will require the consideration of a number of different aspects to ensure the facility will protect the workers and the environment from any hazard. These range from the appropriate primary and secondary containment to reduce the risk from the procedures that are being undertaken, to aspects such as staff training and health surveillance which can be used to identify if there are any undetected breaches in containment. Special attention should be given to the validation of the facility and processes to ensure they are functioning as required, for example it will be necessary to validate the sterilisation techniques for the waste generated.

Overall there needs to be careful consideration of the type of facility that needs to be designed for the handling and processing of samples from extra-terrestrial environments. The type of procedures undertaken should be reviewed and the risks identified to allow for the appropriate measures to be implemented.

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