Contamination

SOURCES OF CONTAMINATION

- Maintaining asepsis is still one of the most difficult challenges to the newcomer to tissue culture.

- Awkwardness during early training can be overcome by experience, but, in certain situations, even the most experienced worker will suffer from contamination.
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- There are several potential routes to contamination including failure in the sterilization procedures for **solutions**, **glassware** and **pipettes**, **dust and spores** in the air in the room, **poorly maintained incubators and refrigerators**, **faulty laminar-flow hoods**, the importation of **contaminated cell lines or biopsies**, and **lapses in sterile technique**.

- The last of these is probably the most significant.

Routes to Contamination

1. Technique Manipulations, pipetting, dispensing, etc.

- Nonsterile surfaces and equipment
- Spillage on necks and outside of bottles and on work surface
- Touching or holding pipettes too low down, touching necks of bottles, inside screw caps
- Splash-back from waste beaker
- Sedimentary dust or particles of skin settling on the culture or bottle;
- hands or apparatus held over an open dish or bottle

2. Work surface

- Dust and spillage

3. Operator hair, hands, breath, clothing

- Dust from skin, hair, or clothing dropped or blown into the culture
- Aerosols from talking, coughing, sneezing, etc.

4. Materials and reagents Solutions

- Nonsterile reagents and media
- Dirty storage conditions
- Inadequate sterilization procedures
- Poor commercial supplier

5. Glassware and screw caps

- Dust and spores from storage
- Ineffective sterilization (e.g., an overfilled oven or sealed bottles, preventing the ingress of steam)

6. Instruments, pipettes

- Ineffective sterilization
- Contact with a nonsterile surface or some other material

7. Culture flasks and media bottles in use

- Dust and spores from incubator or refrigerator
- Dirty storage or incubation conditions.
- Media under the cap and spreading to the outside of the bottle

8. Equipment and Facilities Room air

- turbulence, dust, aerosols

9. Laminar-Flow Hoods

- Perforated filter
- Change of filter needed
- Spillages, particularly in crevices or below a work surface

The commonest example of poor technique is improper use of the laminar-flow hood.

- If it becomes overcrowded with bottles and equipment, the **laminar airflow is disrupted**, and the **protective boundary layer between operator and room is lost**.
- This in turn leads to the entry of nonsterile air into the hood and the release of potentially biohazardous materials into the room.
- In addition, the risk of **collision between sterile pipettes and nonsterile surfaces of bottles, etc., increases**.
- **One should bring into the hood only those items that are directly involved in the current operation**.
- Laminar-flow hoods also must be **maintained regularly**, and the integrity of the filters, ductwork, and cabinets should be checked at least twice a year by a competent engineer.
- The engineer should also check the containment of the workspace, i.e., that internal air does not spill out and outside air does not enter, both of which are dependent on the **internal air velocity and outside turbulence**.

10. Dry incubators

- Growth of molds and bacteria on spillages

11. CO2, humidified incubators

- Growth of molds and bacteria on walls and shelves in a humid atmosphere
- Spores, etc., carried on forced-air circulation

12. Other equipment

- Dust on cylinders, pumps, etc.

13. Mites, insects, other infestations in wooden furniture, or benches, in incubators, and on mice, etc., taken from the animal house

- Entry of mites, etc., into sterile packages

14. Importation of Biological Materials Tissue samples

- Infected at source or during dissection

15. Incoming cell lines

- Contaminated at the source or during transit

TYPES OF MICROBIAL CONTAMINATION

- **Bacteria**, **yeasts**, **fungi**, **molds**, **mycoplasmas**, and occasionally **protozoa**, can all appear as contaminants in tissue culture.

- Usually, the species or type of infection is not important, unless it becomes a frequent occurrence.

- It is only necessary to **note** the **general kind of contaminant** (e.g., bacterial rods or cocci, yeast, etc.), **how it was detected**, the **location where the culture was last handled**, and the **operator's name**.

- If a particular type of infection recurs frequently, it may be beneficial to identify it in order to find its **origin**.

- In general, rapidly growing organisms are **less problematic** as they are often overt and readily detected, whereupon the culture can be discarded.

- **Difficulties arise** when the contaminant is cryptic, either because **it is too small to be seen on the microscope**, e.g., **mycoplasma**, or **slow growing such that the level is so low that it escapes detection**.

- Use of antibiotics can be a common cause of cryptic contaminations remaining undetected.

MONITORING FOR CONTAMINATION

Even in the best laboratories, however, contaminations do arise, so the following procedure is recommended**:**

(1) Check for contamination by eye and with a microscope at each handling of a culture. Check for mycoplasma every month.

(2) If it is suspected, but not obvious, that a culture is contaminated, but the fact cannot be confirmed *in situ*, clear the hood or bench of everything except your suspected culture.
Because of the potential risk to other cultures, this is best done after all your other culture work is finished.
Remove a sample from the culture and place it on a microscope slide.
Check the slide with a microscope.
If it is confirmed that the culture is contaminated, discard the pipettes, swab the hood or bench with **70% alcohol containing a phenolic disinfectant**, and do not use **the hood or bench until the next day**.

(3) Record the nature of the contamination.

(4) If the contamination is new and is not widespread, discard the culture, the medium bottle used to feed it, and any other reagent (e.g., trypsin) that has been used in conjunction with the culture.
Discard all of these into disinfectant, preferably in a fume hood and outside the tissue culture area.

MONITORING FOR CONTAMINATION

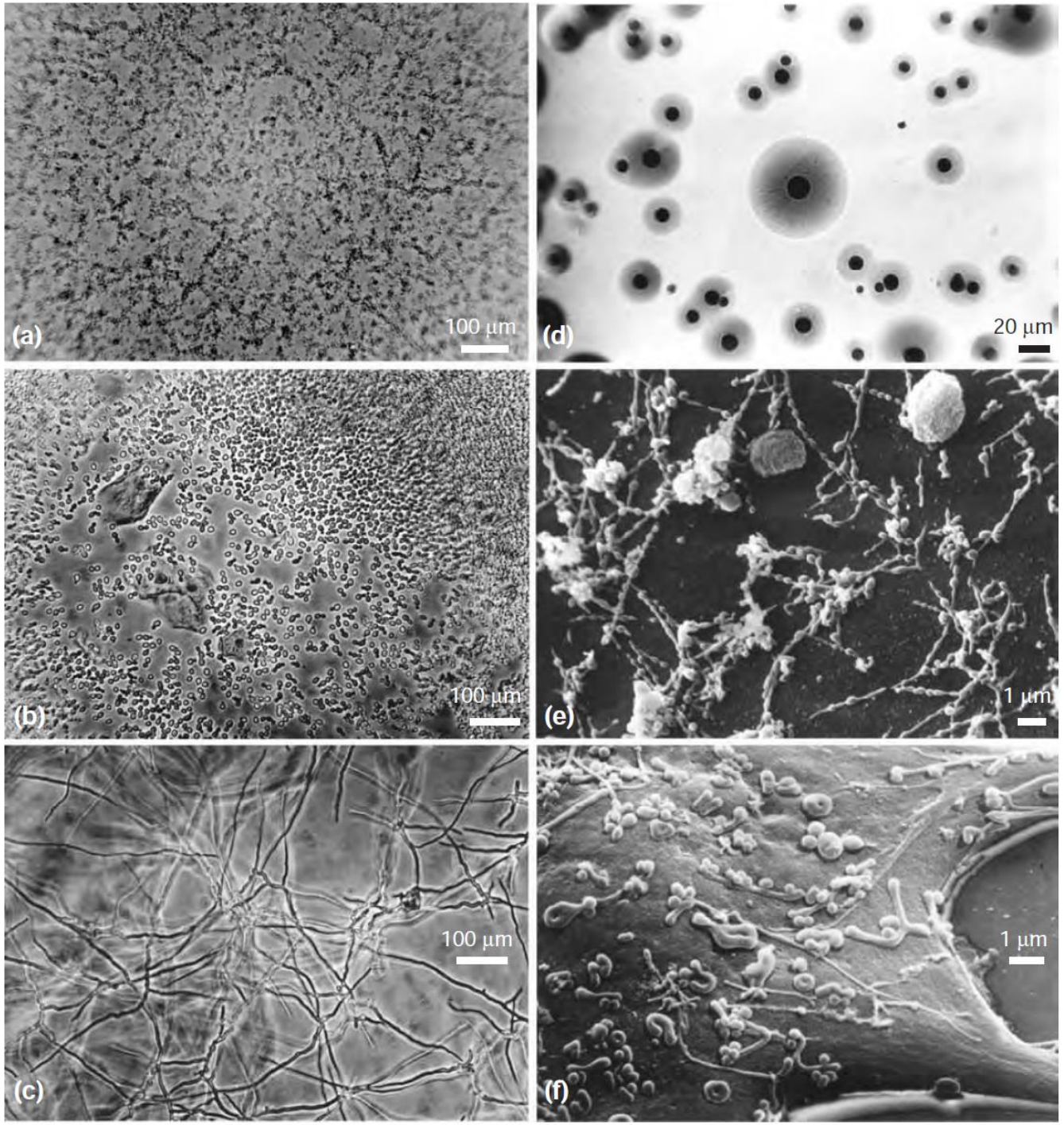
(5) If the contamination is new and widespread (i.e., in at least two different cultures), discard all media, stock solutions, trypsin, etc.

(6) If the same kind of contamination has occurred before, check stock solutions for contamination
(a) by incubation alone or in nutrient broth or
(b) by plating out the solution on nutrient agar.

If (a) and (b) prove negative, but contamination is still suspected, incubate 100 mL of solution, filter it through a 0.2-µm filter, and plate out filter on nutrient agar with an uninoculated control.

(7) If the contamination is widespread, multispecific, and repeated, check
(a) the **laboratory's sterilization procedures** (e.g., the temperatures of ovens and autoclaves, particularly in the center of the load, the duration of the sterilization cycle),
(b) the **packaging and storage practices**, (e.g., unsealed glassware should be resterilized every 24 h), and
(c) the **integrity of the aseptic room** and **laminar-flow hood filters**.

(8) Do not attempt to decontaminate cultures unless they are irreplaceable.



Examples of microorganisms found to contaminate cell cultures.

(a) Bacteria. (b) Yeast. (c) Mold. (d) Mycoplasma colonies growing on special nutrient agar.

(e & f) Scanning electron micrograph of mycoplasma growing on the surface of cultured.

Visible Microbial Contamination

Characteristic features of microbial contamination are as follows:

(1) A sudden change in pH, usually a decrease with most bacterial infections, very little change with yeast until the contamination is heavy, and sometimes an increase in pH with fungal contamination.

(2) **Cloudiness in the medium**, sometimes with a slight film on the surface or spots on the growth surface that dissipate when the flask is moved.

(3) Under a low-power microscope (~×100), spaces between cells will appear **granular** and may **shimmer** with bacterial contamination (Fig. 19.1a). Yeasts appear as separate **round** or **ovoid** particles that may bud off smaller particles (Fig. 19.1b). Fungi produce **thin filamentous mycelia** (Fig. 19.1c) and, sometimes, **denser clumps of spores**.
With toxic infection, some deterioration of the cells will be apparent.

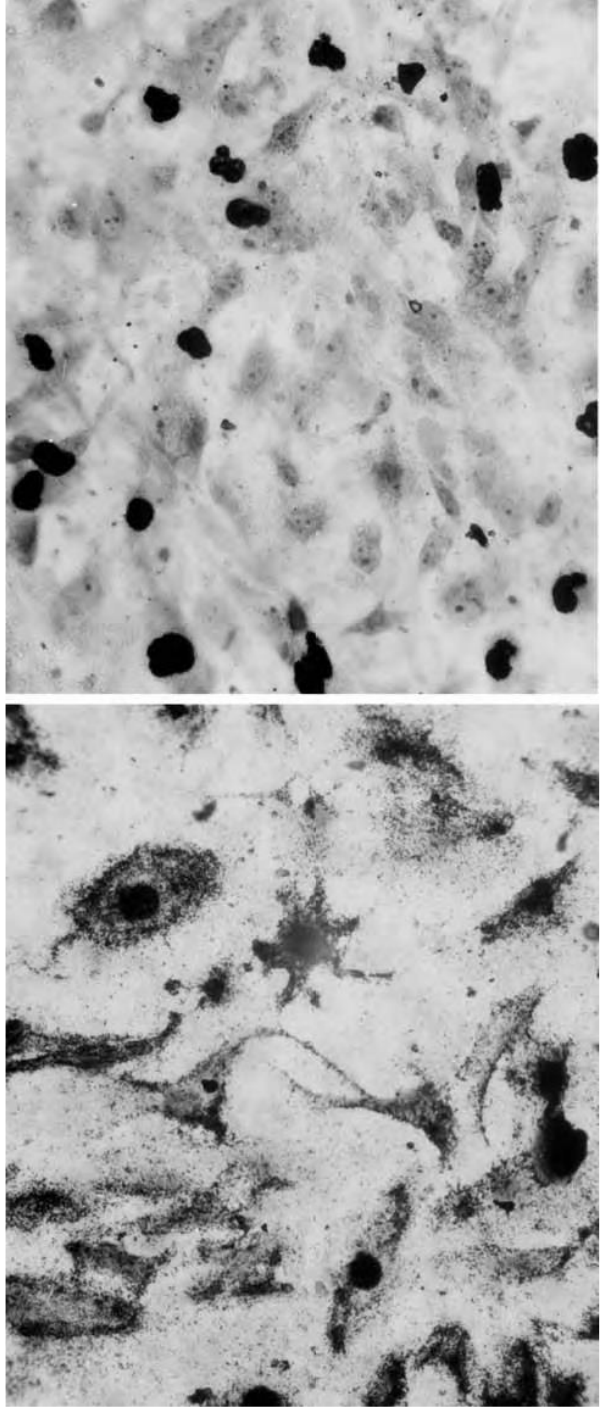
(4) Under high-power microscopy (~×400), it may be possible to resolve individual bacteria and distinguish between **rods and cocci**. At this magnification, the shimmering that is visible in some infections will be seen to be caused by mobility of bacteria. Some bacteria form clumps or associate with the cultured cells.

(5) With a slide preparation, the **morphology of the bacteria** can be resolved at ×1000, but this is not usually necessary. Microbial infection may be confused with **precipitates of media constituents (particularly protein) or with cell debris, but can be distinguished by their regular morphology.** Precipitates may be **crystalline or globular and irregular** and are not usually as uniform in size. If you are in doubt, plate out a sample of medium on nutrient agar.

Mycoplasma

- Detection of mycoplasmal infections (Fig. 19.1d–f) is not obvious by routine microscopy, other than through signs of **deterioration in the culture**, and requires **fluorescent staining**, **PCR**, **ELISA assay**, **immunostaining**, **autoradiography**, or **microbiological assay**.
- Fluorescent staining of DNA by Hoechst 33258 is the easiest and most reliable method and reveals mycoplasmal infections as a **fine particulate** or **filamentous** staining over the cytoplasm at ×500 magnification.
- The nuclei of the cultured cells are also brightly stained by this method and thereby act as a **positive control** for the staining procedure.
- Most other microbial contaminations will also show up with fluorescence staining, so low levels of contamination, or particularly small organisms such as micrococci, can also be detected.

- It is important to appreciate the fact that mycoplasmas do not always reveal their presence by means of macroscopic alterations of the cells or medium. Many mycoplasma contaminants, particularly in continuous cell lines, grow slowly and do not destroy host cells. However, they can **alter the metabolism** of the culture in many different ways.



- Because mycoplasmas **take up thymidine from the medium**, infected cultures show abnormal labeling with [3H]thymidine.

- Immunological studies can also be totally frustrated by mycoplasmal contamination, as attempts to produce antibodies against the cell surface may raise antimycoplasma antibodies.

- Mycoplasmas can alter cell behavior and metabolism in many other ways, so there is an absolute requirement for routine, periodic assays to detect possible covert contamination of all cell cultures, **particularly continuous cell lines.**

Microautoradiograph. This pair of pictures is an example of [3H]thymidine incorporation into a cell monolayer (normal glial cells)
(a) Typical densely labeled nuclei, suitable for determining the labelling.
(b) A similar culture, infected with mycoplasma showing [3H]thymidine incorporation in the cytoplasm.

Monitoring cultures for mycoplasmas

- Superficial signs of chronic mycoplasmal infection include a **diminished rate of cell proliferation**, **reduced saturation density**, and **agglutination** during growth in suspension.

- Acute infection causes **total deterioration**, with perhaps a few **resistant colonies**, although these and any resulting cell lines are not necessarily free of contamination and may carry a chronic infection.

Fluorescence Staining for Mycoplasma

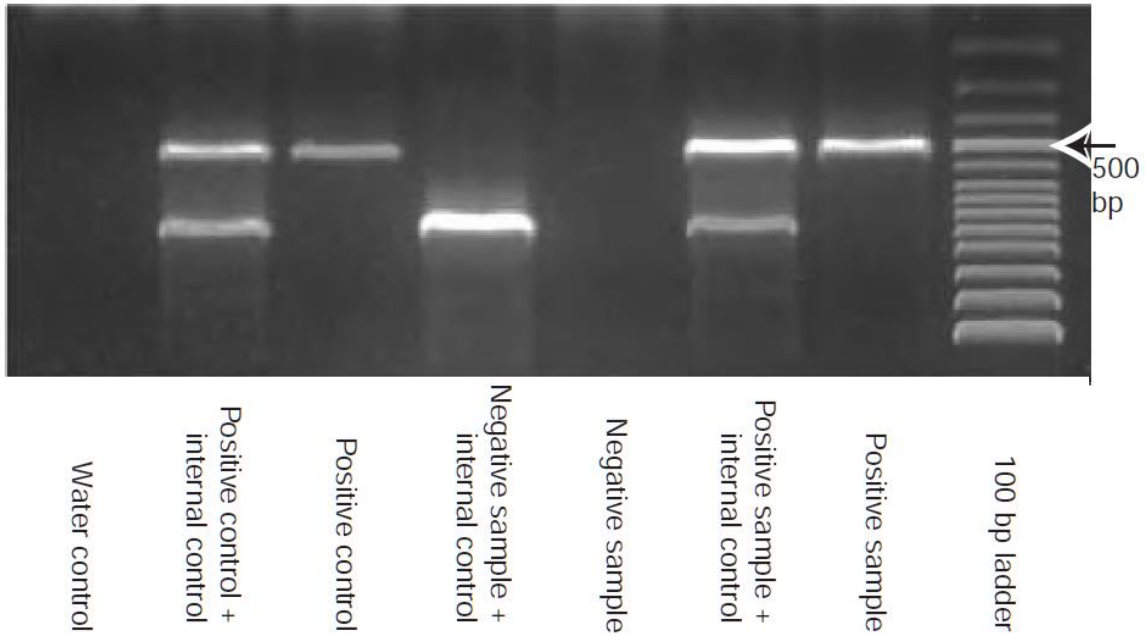
- The cultures are stained with **Hoechst 33258**, a fluorescent dye that binds specifically to DNA.

- Because mycoplasmas contain DNA, they can be detected readily by their characteristic particulate or filamentous pattern of fluorescence on the cell surface and, if the contamination is heavy, in surrounding areas.

- Monolayer cell cultures can be fixed and stained directly, but after centrifugation, the medium from cells growing in suspension will need to be added to an indicator cell (i.e., another monolayer) known to be free of mycoplasma but also known to be **a good host for mycoplasma and to spread well in culture, with adequate cytoplasm to reveal any adherent mycoplasma.** Vero cells, 3T6, NRK, and A549 are all suitable.

- The use of an indicator cell is recommended that it helps to avoid problems with **false positives** arising from debris when cells are assayed just after **thawing** or from **primary cultures**.

- If there is a lot of debris, the medium can be filtered through a sterile 5-µm filter or centrifuged at 100 g



Mycoplasma Detection by PCR. Ethidium bromide fluorescence of PCR products of infected, uninfected, and control cells, electrophoresed on 1.3% agarose. The wildtype mycoplasma bands are about **1000** bp, and the internal control band is almost **510** bp.

PCR for Mycoplasma

The polymerase chain reaction (PCR) provides a very sensitive and specific assay for the direct detection of mycoplasmas in cell cultures with low expenditure of labor, time, and cost, simplicity, objectivity of interpretation, reproducibility, and documentation of results.

Several **primer sequences** are published for both single PCR and with narrow or broad specificity for mycoplasma or eubacteria species.
In most cases, the **16S rDNA sequences** are used as target sequences, because this gene contains regions with more and less conserved sequences.
This gene also offers the opportunity to perform a PCR with the 16S rDNA or an RT-PCR (reverse transcriptase-PCR) with the cDNA of the 16S rRNA.

One of the main problems concerning PCR reactions with samples from cell cultures is the **inhibition of the Taq polymerase** by unspecified substances.
To eliminate those inhibitors, we strictly recommend that the sample DNA be extracted and purified by conventional phenolchloroform extraction or by the more convenient column or matrix binding extraction methods.

Alternative Methods for Detecting Mycoplasma

- **Biochemical.** Among other methods that have been reported for the detection of mycoplasmal infections are methods that detect **mycoplasma-specific enzymes** such as **arginine deiminase** or **nucleoside phosphorylase.**

- **Microbiological culture.** This is a very sensitive method, widely employed in quality control and validation procedures. However, it should only be used with isolation facilities and the appropriate background experience in microbiology, as these microorganisms are quite fastidious, and it will be necessary to culture **live mycoplasma as a positive control**.

- The cultured cells are seeded into mycoplasma broth, grown for 6 days, and plated out onto special nutrient agar. Colonies form in about 8 days and can be recognized by **their size** (~200-µm diameter) and **their characteristic** "**fried egg**" morphology-dense center with a lighter periphery (Fig. 19.1d).
- Commercial kits for microbiological detection (Mycotrim) are available from Irvine Scientific.
- Although using selective culture conditions and examining the morphology of a colony enables the species of mycoplasma to be identified, the **microbiological culture method is much slower and more difficult to perform than the fluorescence technique**. Specific monoclonal antibodies now allow the characterization of mycoplasma contaminations.

- **Molecular hybridization.** Molecular probes specific to **mycoplasmal DNA** can be used in Southern blot analysis to detect infections by conventional molecular hybridization techniques.

- **[3H]thymidine incorporation.** One other method that has been used quite successfully is autoradiography with [3H]thymidine. The culture is incubated overnight with [3H]thymidine with high specific activity and an autoradiograph is prepared. Grains over the cytoplasm are indicative of contamination, which can be accompanied by a lack of nuclear labeling because of trapping of the thymidine at the cell surface by the mycoplasma.

Viral Contamination

- Incoming cell lines, natural products, such as serum, in media, and enzymes such as trypsin, used for subculture, are all potential sources of viral contamination.
- A number of reagents are screened by manufacturers against a **limited range** of viruses, and claims have been made that the larger viruses can be filtered out during processing, but there is no certain way at present to eliminate viral contamination.

- The best way of avoiding it is to ensure that the products are collected **from animals free from known virus infections**. For this, you will need to rely on the quality control put in place by the supplier.

Detection of viral contamination. Screening with a panel of antibodies by immunostaining or ELISA assays is probably the best way of detecting viral infection. Alternatively, one may use PCR with the appropriate viral primers.
Some commercial companies (BioReliance) offer viral screening.

ERADICATION OF CONTAMINATION

Bacteria, Fungi, and Yeasts

- The most reliable method of eliminating a microbial contamination is **to discard the culture and the medium and reagents used with it**, as treating a culture will either be unsuccessful or may lead to the development of an antibiotic-resistant microorganism.

- Decontamination should be attempted only in **extreme situations**, under **quarantine**, and **with expert supervision**.

- If unsuccessful, the culture and associated reagents should be autoclaved as soon as failure becomes obvious.

➢ **The general rule should be that contaminated cultures are discarded and that decontamination is not attempted unless it is absolutely vital to retain the cell strain.**

- In any event, complete decontamination is difficult to achieve, **particularly with yeast**, and attempts to do so may produce hardier, antibiotic-resistant strains.

Eradication of Mycoplasma

- If mycoplasma is detected in a culture, the first and overriding rule, as with other forms of contamination, is that the culture should be **discarded for autoclaving or incineration**.

- In exceptional cases (e.g., if the contaminated line is irreplaceable), one may attempt to decontaminate the culture.

- Decontamination should be done, however, **only by an experienced operator**, and the work must be carried out under **conditions of quarantine**.

- Several agents are active against mycoplasma, including **kanamycin**, **gentamicin**, **tylosin**, **polyanethol sulfonate,** and **5-bromouracil** in combination with UV light.

- Coculturing with **macrophages**, and cytotoxic antibodies can also be effective in some cases.

- However, the most successful agents have been **tylosin**, **Mycoplasma Removal Agent** [MRA], **ciprofloxacin**, and **BM-Cycline** (Roche).

- However, this operation should not be undertaken unless it is absolutely essential, and even then it must be performed in experienced hands and in isolation. It is far safer to discard infected cultures.

Eradication of Viral Contamination

- There are no reliable methods for eliminating viruses from a culture at present; disposal or tolerance are the only options.

Persistent Contamination

Many laboratories have suffered from periods of contamination.

- There is no easy resolution to this problem, other than to follow the previous recommendations in a logical and analytical fashion, **paying particular attention to changes in technique**, **new staff**, **new suppliers**, **new equipment**, and **inadequate maintenance of laminar-flow hoods or other equipment**.

- Typically, an increase in the contamination rate seems from **deterioration in aseptic technique**, **an increased spore count in the atmosphere**, **poorly maintained incubators**, **a contaminated cold room or refrigerator**, or **a fault in a sterilizing oven or autoclave** or **the monitoring of the sterilization cycle**.

- The **constant use of antibiotics** also favors the development of chronic contamination.

✓ **Many organisms are inhibited, but not killed, by antibiotics. They will persist in the culture, undetected for most of the time, but periodically surfacing when conditions change.**

✓ **It is essential that cultures be maintained in antibiotic-free conditions for at least part of the time, and preferably all the time; otherwise cryptic contaminations will persist, their origins will be difficult to determine, and eliminating them will be impossible.**

- A slight change in practices, the introduction of new personnel, or an increase in activity as more people use a facility can all contribute to an increase in the rate of contamination.
- Procedures must remain stringent, even if the reason is not always obvious to the operator, and alterations in routine should not be made casually.

✓ **If strict practices are maintained, contamination may not be eliminated entirely, but it will be reduced and detected early.**

CROSS-CONTAMINATION

- During the development of tissue culture, a number of cell strains have evolved with **very short doubling times** and **high plating efficiencies**. Although these properties make such cell lines valuable experimental material, they also make them **potentially hazardous for cross-infecting other cell lines.**

- The extensive cross contamination of many cell lines with **HeLa** and other rapidly rowing cell lines is now clearly established, but many operators are still unaware of the seriousness of the risk.

The following practices help avoid cross-contamination:
(1) Obtain cell lines from a reputable cell bank that has performed the **appropriate validation of the cell line**.
(2) Do not have culture flasks of more than one cell line, or media bottles used with them, **open simultaneously**.
(3) Handle rapidly growing lines, such as HeLa, **on their own and after other cultures**.
(4) Never use the same pipette for different cell lines.
(5) Never use the same bottle of medium, trypsin, etc., for different cell lines.
(6) Do not put a pipette back into a bottle of medium, trypsin, etc., after it has been in a culture flask containing cells.
(7) Add medium and any other reagents to the flask first, and then add the cells last.
(8) Check the characteristics of the **culture regularly**, and suspect any sudden change in morphology, growth rate, or other phenotypic properties.

- Cross-contamination or its absence may be confirmed by **DNA fingerprinting**, **DNA profiling karyotype** or **isoenzyme analysis**.
- It cannot be overemphasized that cross-contaminations can and do occur. It is essential that the preceding precautions be taken and that cell strain characteristics be checked regularly.