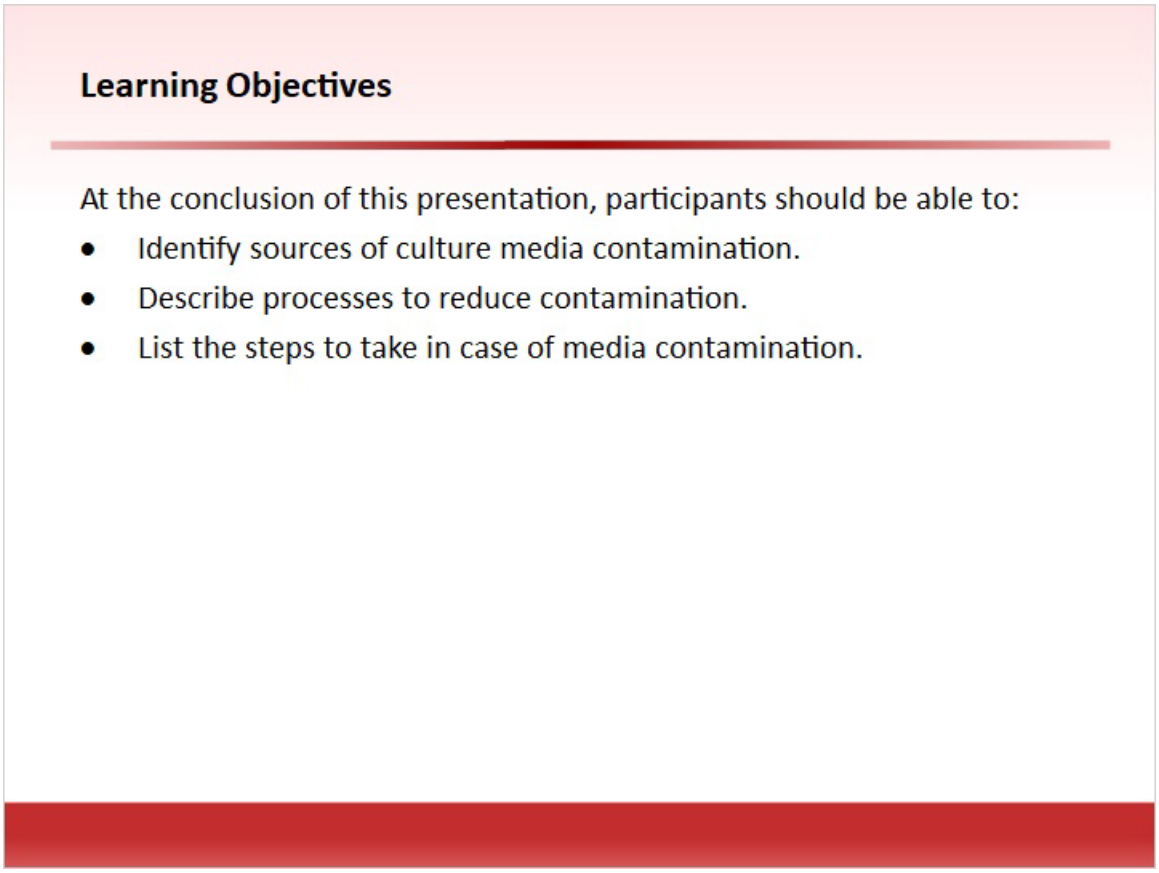
LABCC100 Lesson 14

1.1 Microbial Contamination of IVF Cultures

Notes:

Welcome to the American Society for Reproductive Medicine's eLearning modules. The subject of this presentation is Microbial Contamination of IVF Cultures.

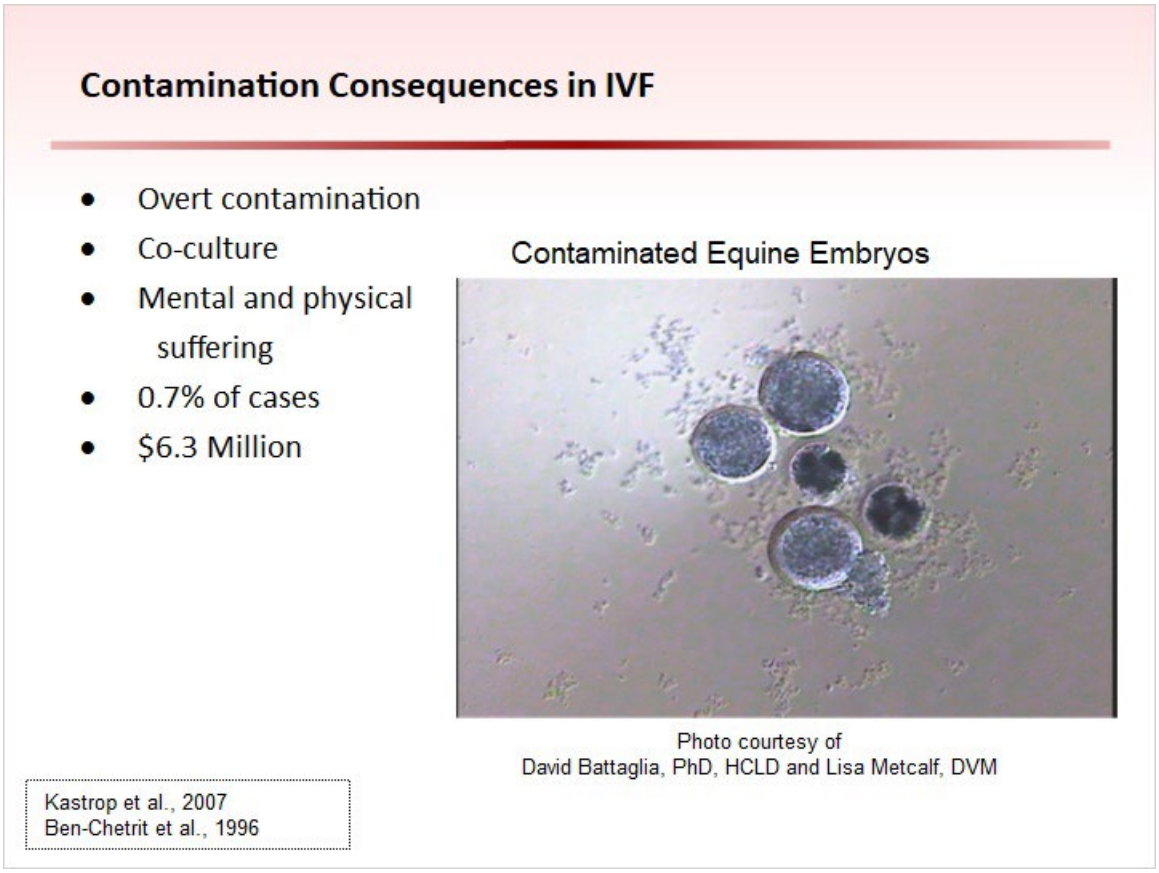
1.2 Learning Objectives



Notes:

At the conclusion of this presentation, participants should be able to: identify sources of culture media contamination, describe processes to reduce contamination, and list the steps to take in case of media contamination.

1.3 Contamination Consequences in IVF



Notes:

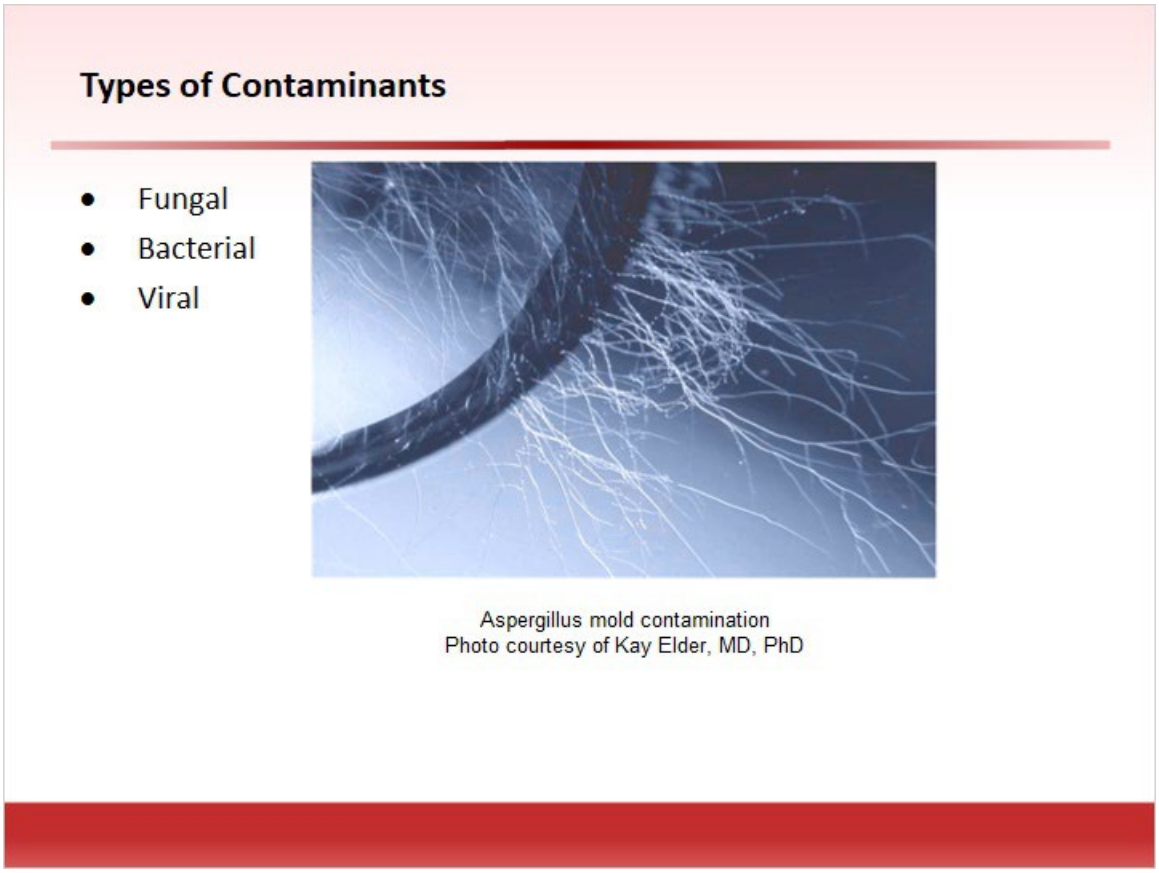
One of the most difficult experiences an embryologist can face is the observation of an overt contamination of culture media: embryos appear as “burnt cinders” and are surrounded by a flocculence of debris. These embryos are degenerate. Not only should all remaining embryos be suspected of being contaminated, but it is highly questionable whether any of the potentially contaminated embryos should even be transferred.

Another contamination scenario is seeing growing embryos surrounded by a mesh of “fungal threads” and possibly what looks like a “floating cotton ball.” These “co-cultured” embryos are not dead and degenerate, but is it safe to transfer them into patients?

It is amazing that so little contamination is observed in IVF when embryos are cultured in a growth medium containing added fluids (such as follicular fluid and semen) with bacteria, viruses, and fungi. Even the use of antibiotics (usually gentamicin) in IVF culture media should do little as it is not effective against gram-negative bacteria, fungi, or viruses.

Not only is microbial contamination an issue due to the deleterious effect on embryos but these pathogens could then be introduced into the patient's uterus, resulting in a potentially life-threatening infection. A few researchers have measured the incidence of contamination events in IVF at about 0.7% of IVF cases. In the United States, even this level of incidence could result in a cost of over $6 million per year as well as immeasurable mental suffering. It is important to reduce the incidence of contamination, but first the origin of these microorganisms that contaminate these cultures must be identified.

1.4 Types of Contaminants



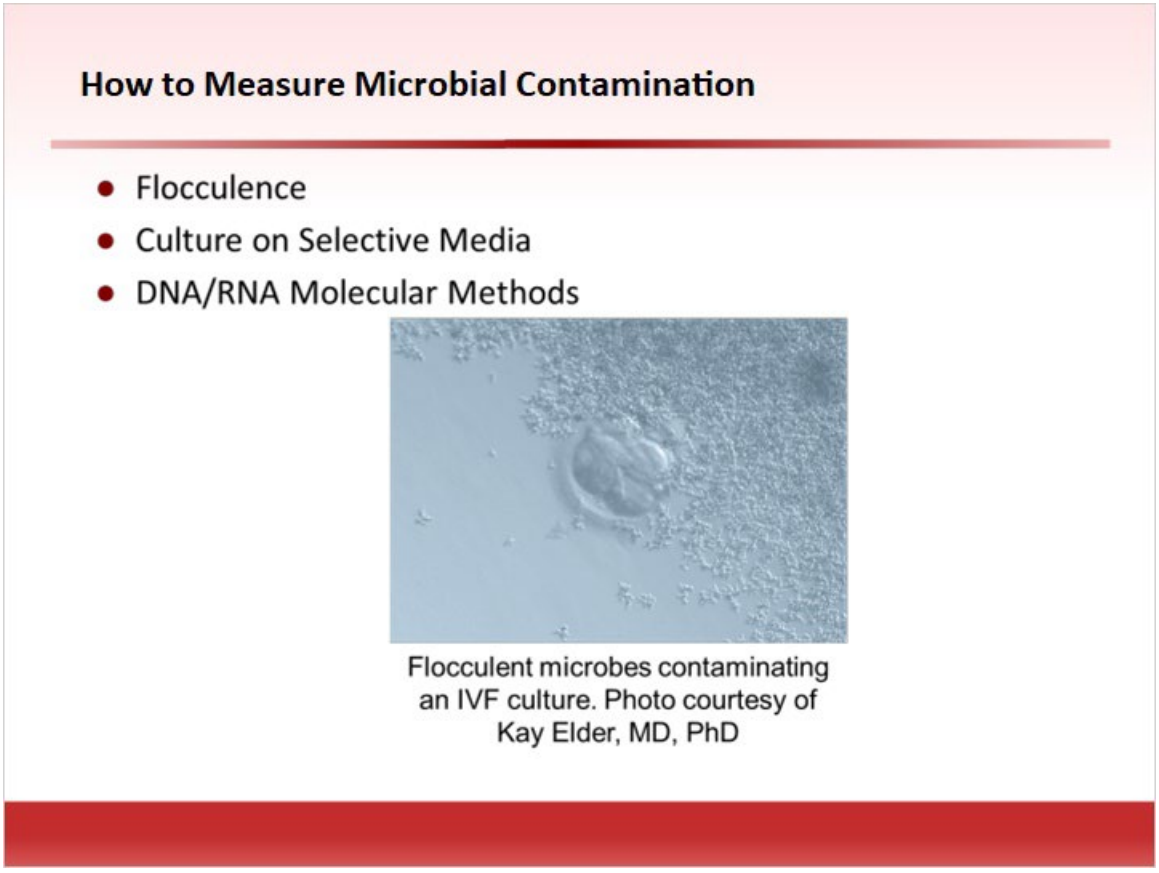
Notes:

There are three major types of culture microbial contaminants: fungi, bacteria, and viruses. The most common fungal contaminants in tissue cultures are *Aspergillus* (aerobic mold) and *C. albicans* (yeast). Common bacterial contaminants include *E. coli*, *mycoplasma*, *Staphylococcus,* and diphtheroids (gram-positive rods). Both bacteria and fungi have been identified in IVF cultures. Although viruses are another potential microbial contaminant, almost nothing is known about their role in embryo culture.

There have been no known viral surveys identifying viral contaminants, nor is there much research on the role of viral contaminants in IVF cultures.

While bacterial contamination often results in the demise of the embryo, if fungal contamination is caught early enough the embryos are usually not harmed. In 1 report, 7 cases of fungal contamination resulted in pregnancies in all of the seven cases (Ben-Chetrit et al., 1996). Embryos were merely washed free of the fungi and were then transferred. It may be that when fungi do harm, it is by either depletion of energy substrates or due to a buildup of harmful metabolites and that as long as the media is replaced and fungal contaminants are removed, the embryos will continue to grow.

1.5 How to Measure Microbial Contamination



Notes:

There are several methods for measuring microbial contamination, and some methods are more sensitive than others. The most obvious measure of contamination (but clearly not the most sensitive) is observation of the flocculent material in the embryo culture drops. Two other methods often used for determining contamination are culturing on

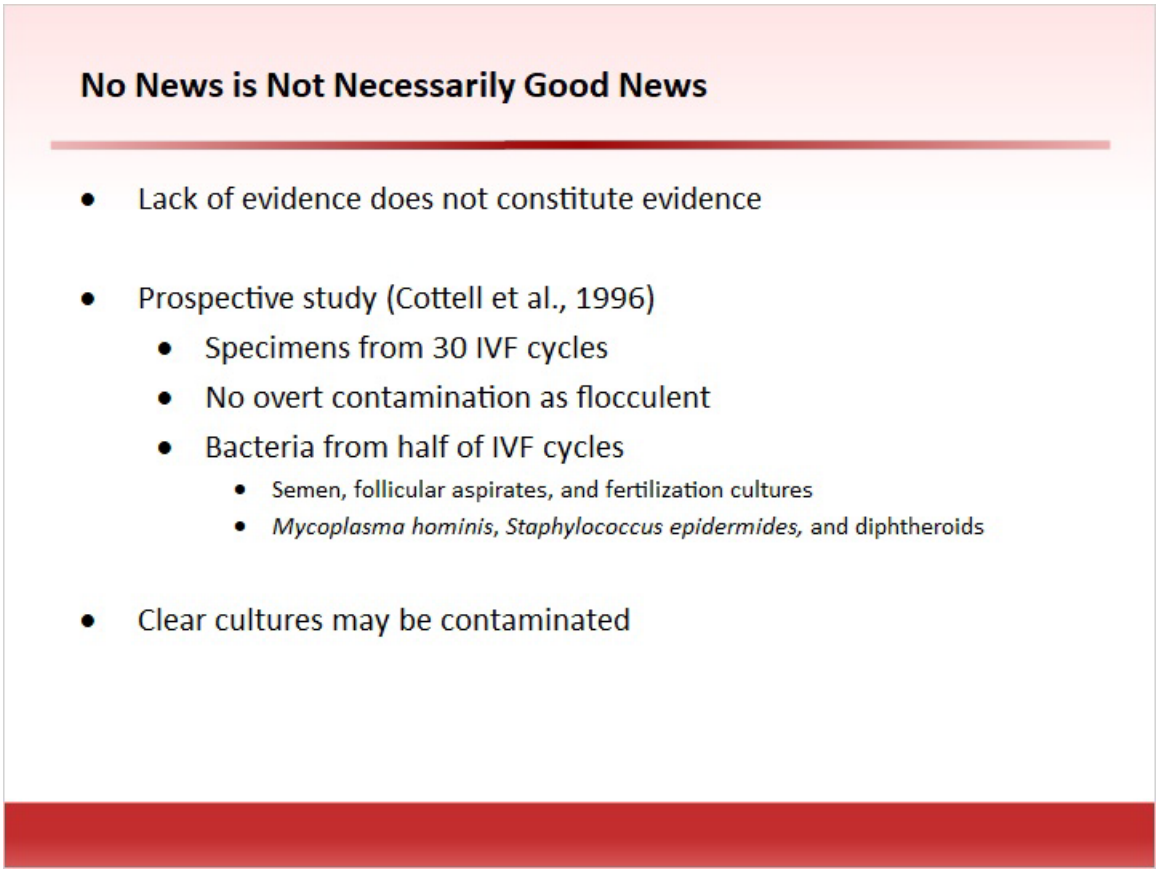
selective media or using molecular technologies (such as polymerase chain reaction [PCR]) to detect either DNA or RNA sequences peculiar to each organism.

The traditional method for measuring microbial contamination is to take some of the suspected media and streak it on a plate with growth media. If an organism is present it will result in streaks of colonies on the plate. The type of organism can then be determined by noting what type of media it grows on and by the morphological characteristics of the colonies (shape and color). However, lack of growth does not mean there are no organisms present. It may just mean that the organism present cannot grow on that particular medium or that the particular conditions (too much oxygen, for example) are inadequate for that organism's growth.

Some bacteria (and most viruses) are difficult to isolate and identify by culturing. For example, obligate anaerobes will not culture in media that is exposed to oxygen. Thus, failure to find them in semen using an agar plate cultured in air does not indicate that the bacteria is not in the specimen. Plating of microorganisms on growth agar will also not allow the detection of organisms that have died. If a suspect culture is isolated for testing but there is a delay in when it is sent out for identification and the conditions that promoted its growth are not maintained, the dead microorganisms will not be detectable unless a molecular method is used. The most sensitive methods for determining the presence of microbes are where molecular techniques such as PCR are used to detect sequences particular to a genus or species.

These considerations are also important to keep in mind if you suspect a contamination event and you are sending culture media to a microbiology lab to help determine the potential cause.

1.6 No News is Not Necessarily Good News



Notes:

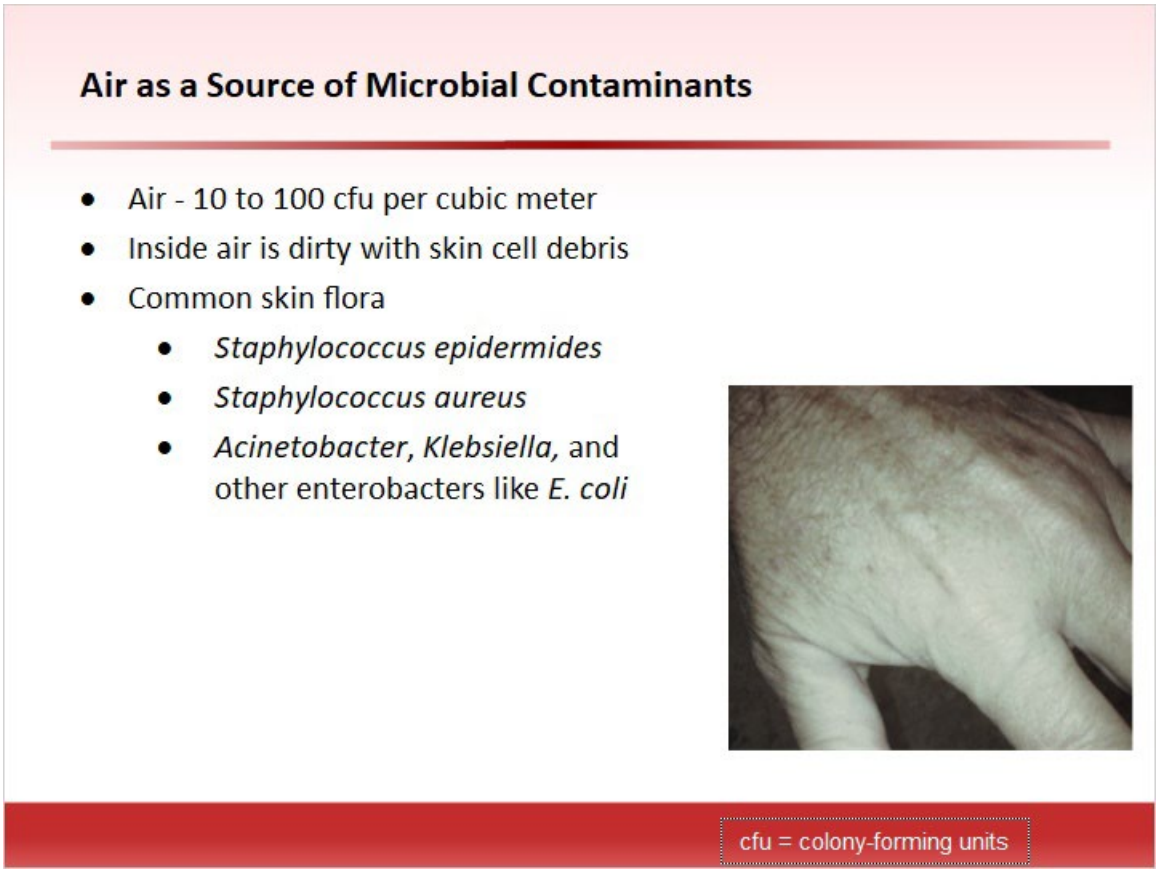
Lack of an overt contamination (flocculence) in IVF cultures does not indicate lack of microbial contamination.
In one prospective study (Cottell et al., 1996), specimens from 30 IVF cycles were examined for contaminants using culture on selective media. Despite finding no overt contamination as flocculent, they cultured bacteria from half of the IVF cycles. They found bacteria in semen, follicular aspirates, and fertilization cultures. The most common organisms found were *Mycoplasma hominis*, *Staphylococcus epidermides,* and diphtheroids. One can conclude from this, that the clear cultures seen routinely might still contain contaminating organisms, and these organisms could be affecting the growth of embryos.

1.7 Sources of Contamination

Notes:

When an embryologist observes overt contamination, the first response is often one of panic, “Where did this come from? Did I do this?” There are several potential sources for these microbes including the environment, laboratory supplies, techniques of physicians, nurses, medical assistants, embryologists or andrologists, and the patient's own contributions to the IVF cycle-ova bathed in follicular fluid and sperm bathed in semen. Each of these sources will now be examined.

1.8 Air as a Source of Microbial Contaminants

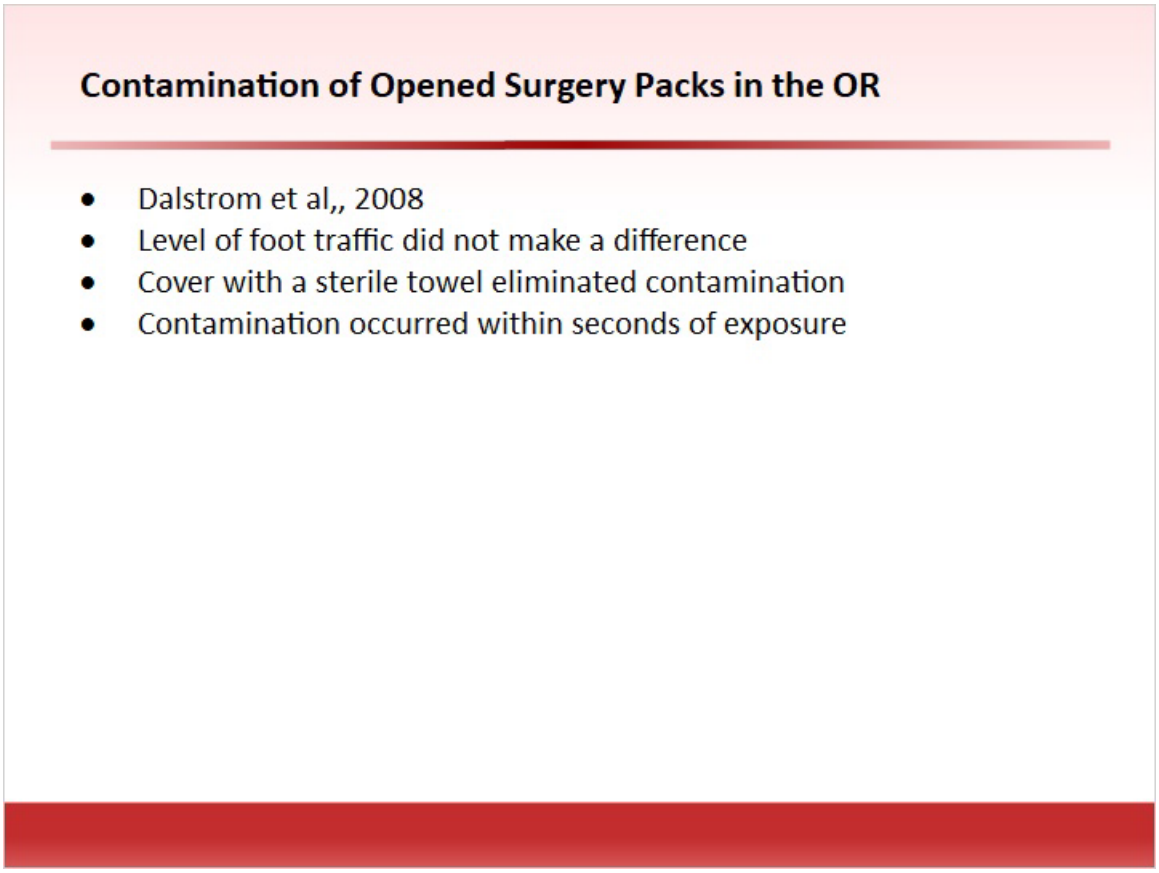


Notes:

Air contains bacteria, fungi, and viruses. The outside air may contain 10 to 100 colony-forming units of microorganisms per cubic meter, and inside air is even dirtier. Inside air is probably dirtier due to the fact that human skin literally “snows” sloughed-off dead skin that is covered with carpets of bacteria. It has been estimated that humans lose 30,000 to 40,000 skin cells every minute.

The shedding of skin probably accounts for much of the contamination of floors with bacteria, while the walls and ceilings seldom are contaminated. The dust found inside consists largely of skin cells and their accompanying bacteria. The most common skin flora are *Staphylococcus epidermides*, *Staphylococcus aureus*, *Acinetobacter*, *Klebsiella,* and other enterobacters like *E. coli*. Air is a potential source of contamination, especially if someone reaches over open catheters, needles, dishes, or other sterile items and a flake of skin happens to rain down.

1.9 Contamination of Opened Surgery Packs in the OR



Notes:

An interesting example of how simple exposure of sterile items in a positive-pressure operating room setting affects sterility was demonstrated by Dalstrom and colleagues who opened 45 sterile trays in an operating room. One third of the trays were opened in an OR suite that was then locked (no foot traffic), one third in a room with foot traffic of a single person going in and out every 10 minutes, and another set of 15 trays where the tray was covered with a sterile towel, and then the room was locked. Only those trays that were opened, covered by a sterile towel and then locked remained uncontaminated. Four percent of the trays were contaminated after 30 minutes and 30% after 4 hours of exposure to these conditions. Interestingly, 3 trays were found to be contaminated immediately after opening.

This study demonstrates that the major factor responsible for contamination is an opened container and that the presence of people is not necessary for this contamination.

1.10 Laboratory Supplies as a Source of Contamination

Notes:

There is at least one example in the past where oil used by IVF clinics was found to be contaminated with fungi at the air-oil interface in the bottle. Microorganisms do not have to be alive in order to harm embryos.

Most commercial suppliers of media, media supplements, or disposable plasticware must meet stringent sterility requirements that greatly reduce the risk of these items bringing contaminants into the laboratory. Still, it is possible that improper storage of IVF supplies or damage during packaging or storage could result in inadvertent contamination. Improper use of these items could also lead to contamination. For example, failure to open sterile packs properly or storing them in a location that gets wet could easily result in contamination of devices and consequently contamination of embryo cultures. Improper handling of any of these sterile devices, such as allowing the outer wrap of a pipette to touch the sterile pipette, could result in the contamination of the culture medium.

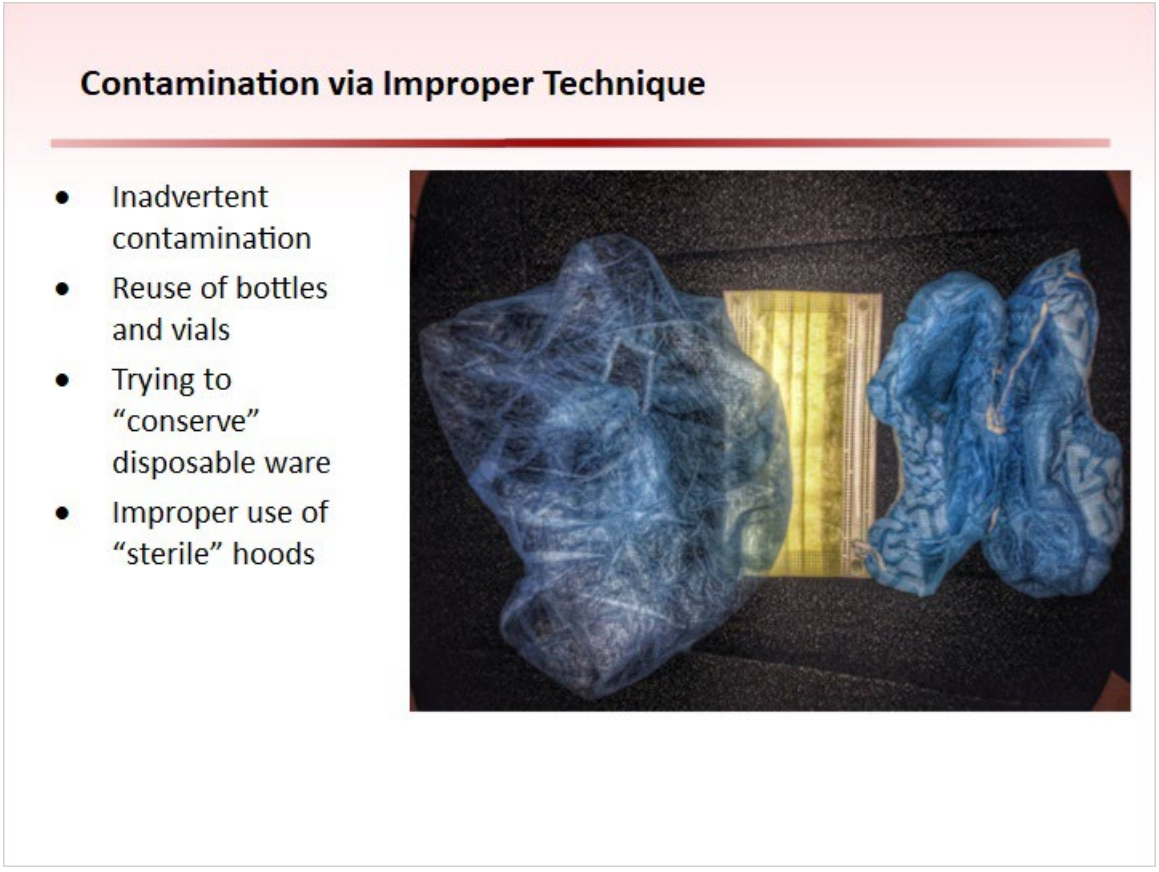
One must also pay attention to the method of sterilization used for materials as some of these sterilization methods (ethylene oxide, for example) can produce residual toxicants.

For example, a catheter should not be sterilized with ethylene oxide, as this could have deleterious effects on embryos.

It is important that all items from manufacturers and clinics be sterilized properly in order to avoid the introduction of contaminants. Proper procedures for autoclaves and dry sterilizers must be followed, including regular monitoring of the effectiveness of these sterilizers using biological methods. A common method to sterilize liquids such as media and some sera is the use of filters. Most filters used are 0.22 microns in pore diameter. Unfortunately, as will be discussed later, the bacterium *Mycoplasma* can pass through these pores due to its small size and lack of a cell wall.

Even the products of dead microorganisms (the lipopolysaccharide membrane of gram-negative bacteria) can result in the destruction of embryos. For this reason, most fluids and contact materials are tested for endotoxins and must pass specific quality-control standards for endotoxins.

1.11 Contamination via Improper Technique



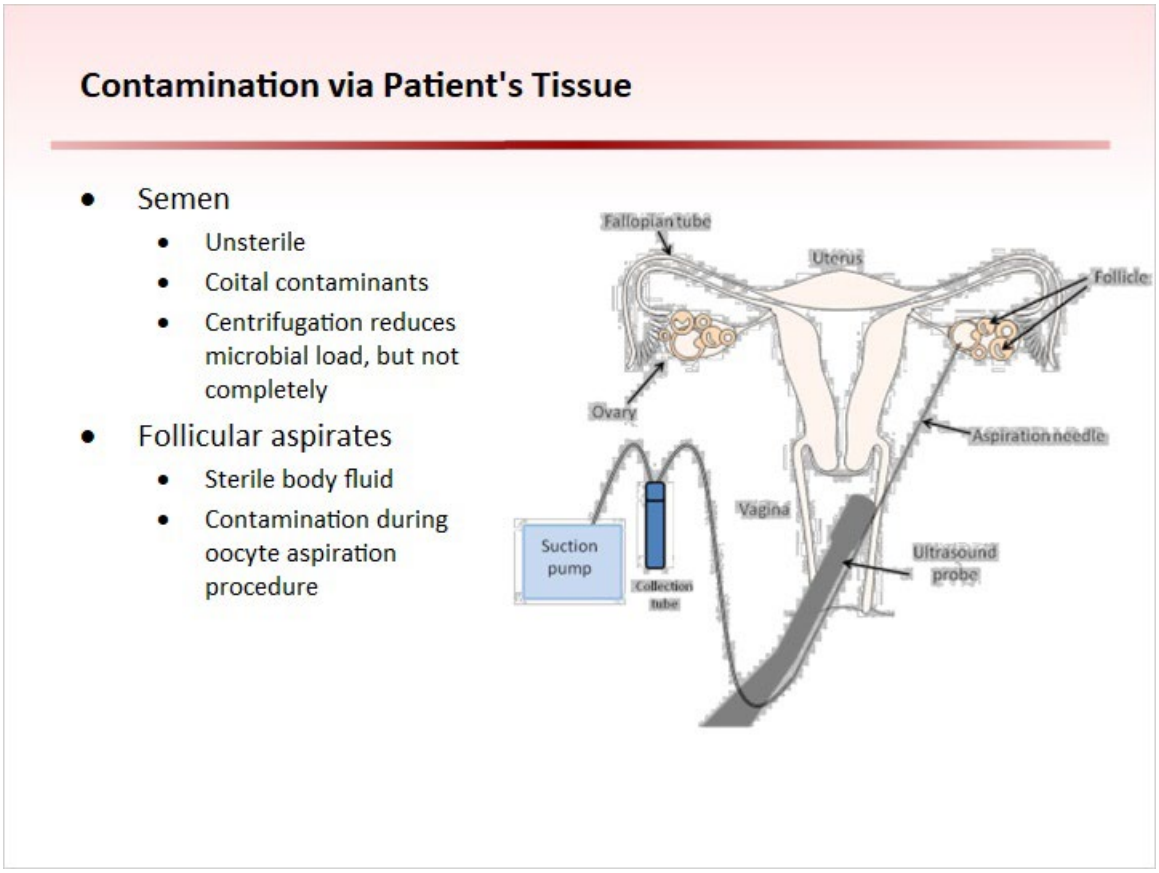
Notes:

Many points exist in the IVF procedure where improper handling could result in contamination. This includes inadvertent contamination via not using sterile technique or inadvertently touching a sterile item to a non-sterile item. For example, a nurse preparing the needle guide during setup for follicular aspiration of ova could inadvertently touch the bare ultrasound probe while placing the probe cover on and could then contaminate the aspiration needle by touching the needle guide. When the needle is then placed through this needle guide, these contaminants could be passed on to the follicular fluid that is aspirated.

Improper technique in the reuse of vials or other liquids is also a potential source of contaminants and can be eliminated by using single-use media. Every time a bottle or vial is accessed, another chance for contamination occurs. Sometimes, in an effort to cut costs, the risks for contamination are increased. But, when one considers the cost of one contamination event, this is not prudent.

Two types of flow hoods are common to IVF laboratories. The biosafety cabinet is designed to protect the laboratory worker and the environment from pathogens. The Class I hood is designed to protect the worker but is not designed to protect contamination of items in the cabinet. A Class II hood is designed for both types of protection (worker and products). One of the problems with any hood is that the insertion of items (like a microscope) into the air flow can result in contaminating that air. Some problems with the use of these hoods in human IVF laboratories are noise, inability to properly sterilize due to volatiles used for sterilization, and increase of evaporation and osmolality when using small drops for culture.

1.12 Contamination via Patient's Tissue

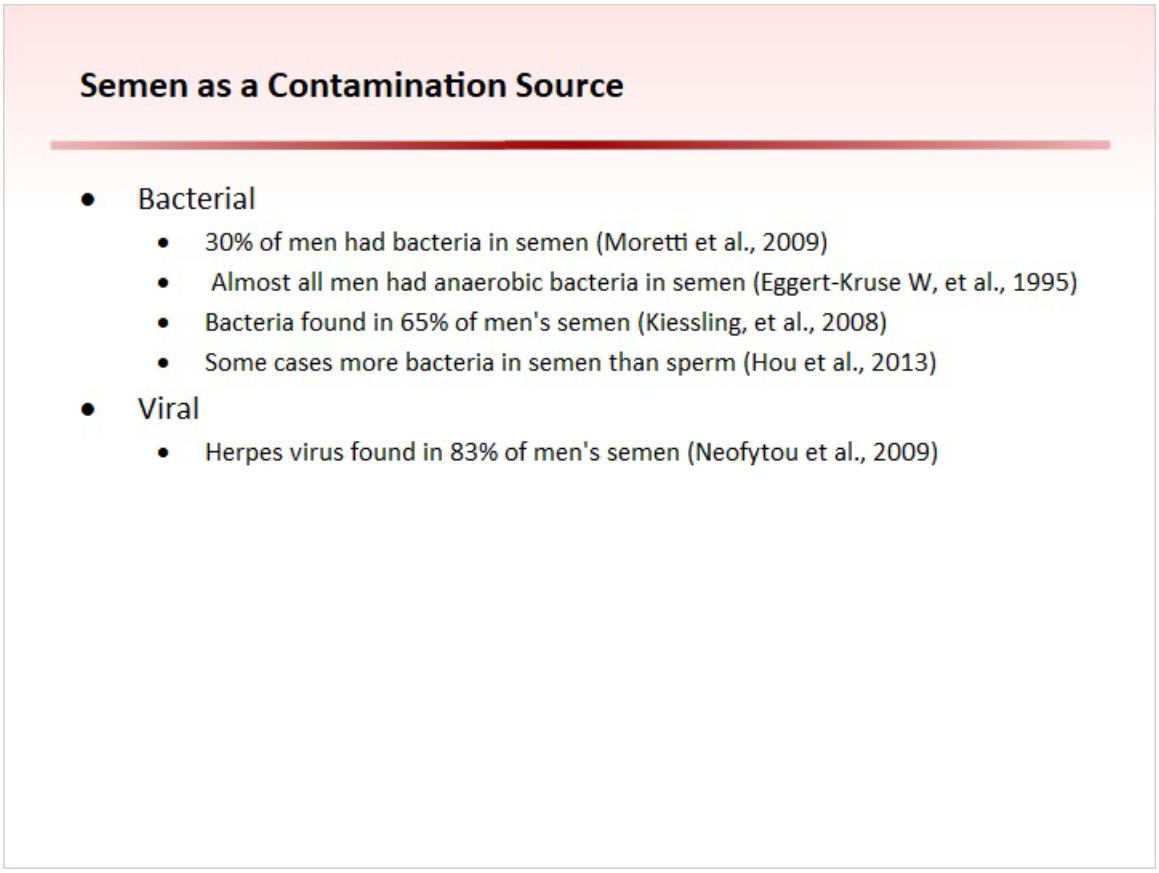


Notes:

Two potential sources of microbial contamination in IVF come from the patients' own fluids: semen and follicular aspirates. Semen is not sterile but usually has low levels of bacterial and viral contaminants. The most obvious sources of bacteria in semen are ascending microorganisms. These can occur during coitus with the female partner or via the environment. Upon ejaculation, the microbes in the semen could then be passed on to the culture media during the addition of sperm to the fertilization drop. Although centrifugation through gradient columns will reduce the microbial load of contaminated semen, it will not necessarily reduce it to zero, especially if the microbes bind to the sperm.

Follicular fluid, often considered a sterile body fluid, can be contaminated when follicles are aspirated. The needle has to pass through the vaginal wall, which is most likely the source of the contaminants. The vagina acts as a culturing environment to many microorganisms.

1.13 Semen as a Contamination Source



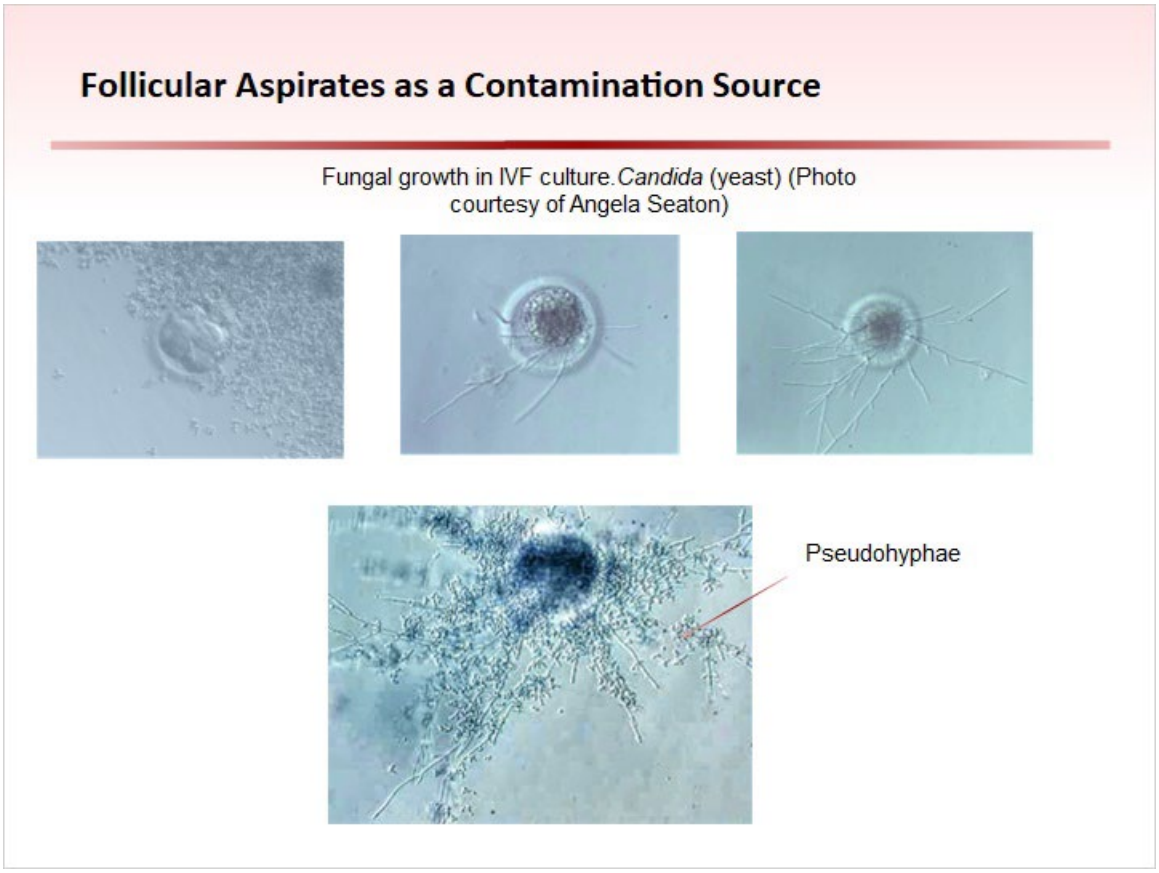
Notes:

Bacteria appear to be a common contaminant of semen. One study found that about 30% of men (n=1,256) had bacteria cultured from their semen. This is probably an underestimation, as fastidious and anaerobic organisms would not have been identified using the culturing method of this study. *E. faecalis* and *E. coli* were the most common bacteria identified. Another study reported that almost all ejaculates they examined for anaerobic bacteria were positive. Kiessling's study in 2008 found that the use of new molecular techniques for identifying microorganisms detected bacteria in the semen of 65% of men. Anaerobic, gram-positive cocci dominated, and the investigators concluded that these bacteria were the result of male genitourinary infections and not from commensal species.

A recent survey of microorganisms in semen used a sensitive method of DNA sequencing of the 16S rRNA gene to determine the types of microorganisms found in semen. This survey found that most semen samples have high numbers of bacteria present. In some cases, there were more bacteria than sperm in the semen. In another study, 13% of men were positive for *Chlamydia trachomatis* DNA in semen.

At least one study has examined semen for herpes virus and found that 83% of their study group comprised of 172 men had a herpes virus. This included human herpesvirus 6 (HHV6), Epstein-Barr virus (EBV), and cytomegalovirus (CMV). It appears that most semen is not sterile and contains bacteria and/or viruses.

1.14 Follicular Aspirates as a Contamination Source



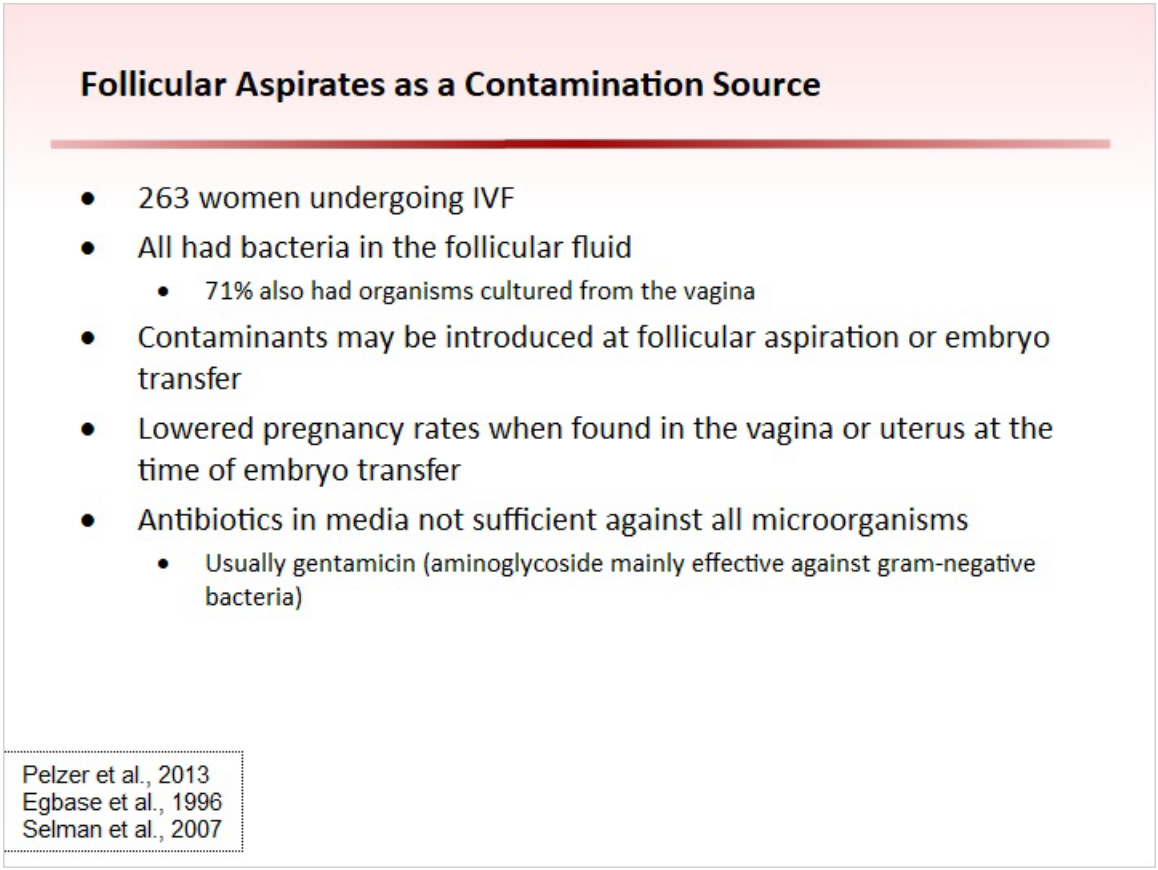
Notes:

Many clinicians are surprised when an IVF culture becomes overtly contaminated with bacteria or fungi. However, this condition may be the rule rather than the exception. It may be that all cultures become contaminated from both the follicular aspiration and the semen, but either the medium does not provide the necessary nutrients for growth or the intense washing of ova and embryos dilutes out the microorganisms sufficiently so they are not visible as flocculence.

The biggest source of culture contamination most likely comes from follicular aspirates.

The vagina is full of bacteria and fungi. One of the most common microorganisms found in the vagina is *Lactobacillus*-found in almost all women. Other bacteria found include *Staphylococcus epidermides*, *Clostridium perfringens* and *Ureaplasma*. The aspiration needle must repeatedly pass through this microorganism-populated area during aspiration of the follicles.

1.15 Follicular Aspirates as a Contamination Source



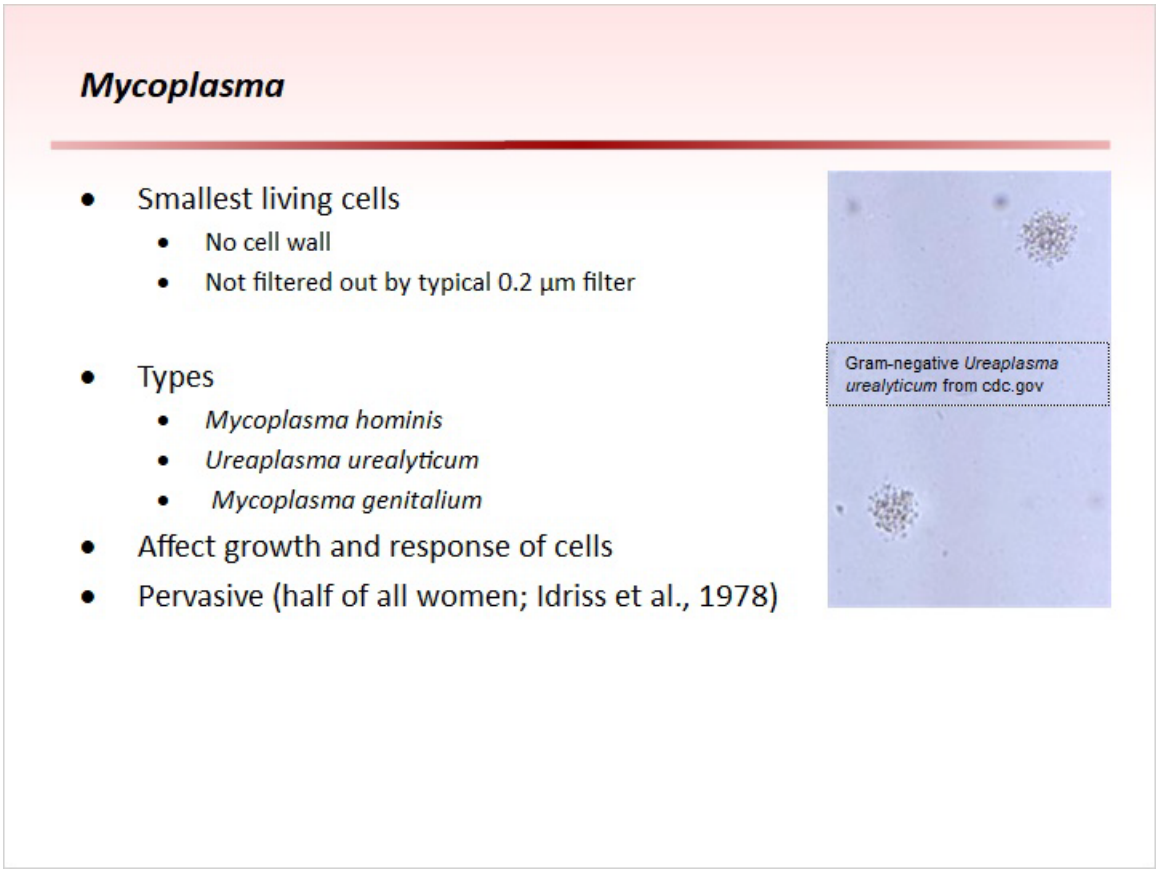
Notes:

Pelzer et al. (2013) used traditional culturing techniques to examine the follicular fluid of women undergoing ova retrieval for the presence of bacteria. They found bacteria in the follicular fluid of all 263 women undergoing ova retrieval for IVF. Seventy-one percent of the time these organisms were cultured from the vagina also, indicating that the vagina might be the source. This raises the question about whether some of these organisms might be introduced into the reproductive tract during follicular aspirations or embryo transfers. Some studies indicate that these contaminants can also result in lowered pregnancy rates when found in the vagina or uterus at the time of embryo transfer. Regardless of the source, follicular fluid is not a sterile fluid and could be one of the

major sources of media contamination.

That the incidence of overt microbial contamination is only about 0.7% is amazing when one considers that both non-sterile semen and follicular fluid are added to a nutrient culture media that often contains a low dose of a single antibiotic. In most cases the antibiotic gentamicin, an aminoglycoside that is mainly effective against gram-negative bacteria. However, the type and dose of gentamicin in media is not sufficient to inhibit or kill all microorganisms.
It is surprising that most IVF cultures do not result in overt contamination events.

1.16 Mycoplasma



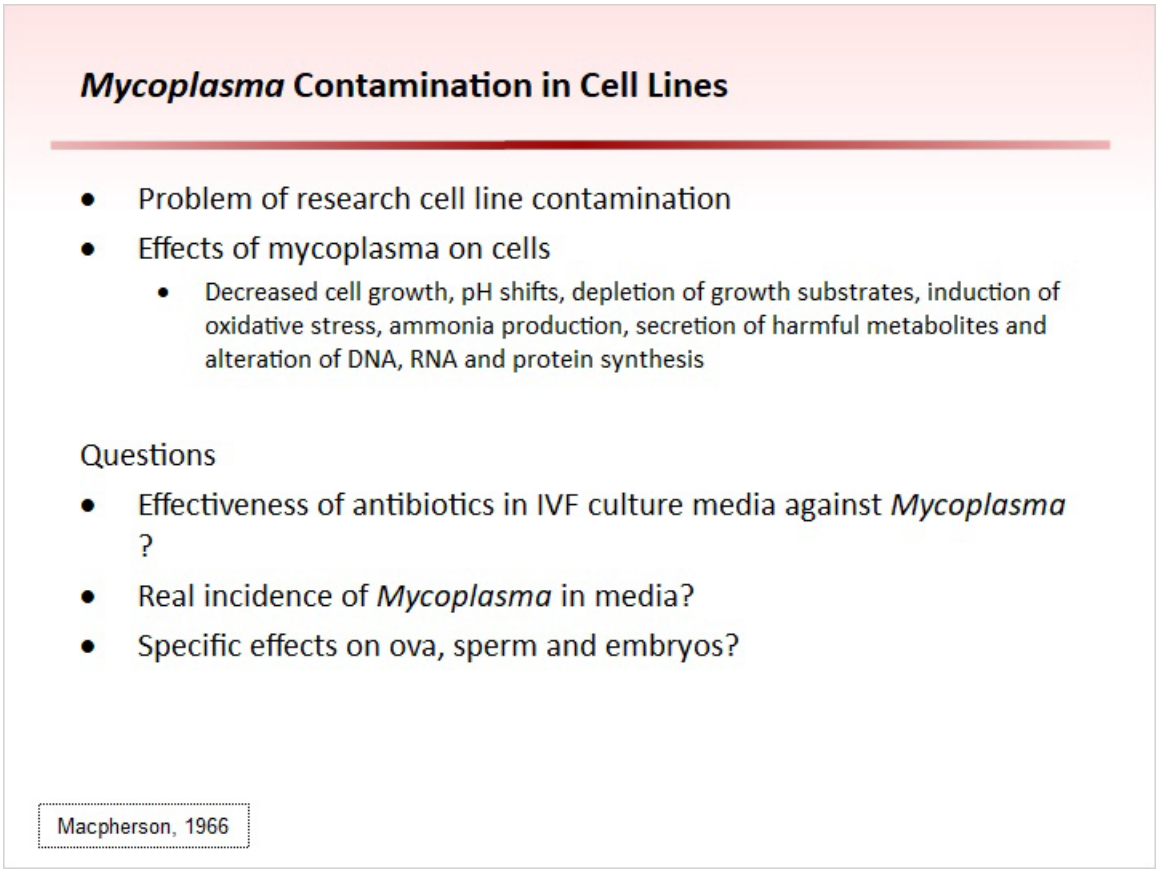
Notes:

Examining just one bacterium might help to see what potential effect these microbes could have as a contaminant in embryo cultures and may tell much about these unseen risks.

Mycoplasma are the smallest living cells. They are not affected by most antibiotics nor

are they filtered out using traditional filter pore sizes. Because *Mycoplasma* lack a cell wall, they are not affected by beta-lactam antibiotics (such as penicillin) that target the cell wall. Lack of a cell wall and their small size make them difficult to filter out. The typical 0.2 µm filter used for media filtration will not filter out *Mycoplasma*. (A 0.1 µm pore size is necessary to filter them out.) *Mycoplasma hominis* and *Ureaplasma urealyticum* are found in the cervix or vagina of more than half of all women. *Mycoplasma hominis* can result in infertility by causing pelvic inflammatory disease (PID), urogenital infections, and septicemia. *Mycoplasma genitalium* has been implicated in urethritis in males. The risk for a follicular aspirate to contain *Mycoplasma* is high, as one study found that 55% of infertile women tested positive for the organism.

1.17 Mycoplasma Contamination in Cell Lines



Notes:

In the early 1960s, it was discovered that most research cell lines of cell cultures were contaminated by *Mycoplasma* (Macpherson, 1966). Contamination of cell cultures in research resulted in the invalidation of many experiments due to the modifications mycoplasma had on transcription, translation, etc. As a result, a lot of time and money

have been spent trying to remove *Mycoplasma* contamination from cell lines and keeping them free of contamination.

Effects of *Mycoplasma* on cells include decreased cell growth, pH shifts, depletion of growth substrates, induction of oxidative stress, ammonia production, secretion of harmful metabolites, and alteration of DNA, RNA, and protein synthesis.

It is important to note that most antibiotics used in IVF culture media are not effective against an organism such as *Mycoplasma* . The level of gentamicin most often found in embryo culture media is 1/20th of that necessary to effectively inhibit its growth.

In summary, it appears that *Mycoplasma* organisms are present in patients; they are not filtered out of media using traditional filtration methods; antibiotics used are ineffective in inhibiting its growth; and this microorganism can negatively affect the growth and development of eukaryotic cells. What is not known is the real incidence of *Mycoplasma* in media or its specific effects on ova, sperm, and embryos.

1.18 How to Reduce Contamination

How to Reduce Contamination

- Reduce dust (contains lots of skin cells)
- High-efficiency particulate absorption (HEPA)
- Decrease air flow
- General cleanliness
- Scrubs
- Technique
- Hoods
- Masks
- Sticky pads
- Training
- Pipetting

Notes:

Several methods are available to reduce the risk of contamination of embryo cultures. Two of the most common potential contaminants in the laboratory are skin and dust. Skin is colonized with plaques of *Staphylococcus epidermides* and *Corynebacterium*. Simple cleaning of floors and walls can reduce this bacterial load by 80%. Embryologists and clinicians must also be aware that our skin rains down from personnel reaching over dishes and may result in their inadvertent contamination.

To help reduce the load of microorganisms, most laboratories have installed particle filters using high-efficiency particulate absorption (HEPA) hoods, and sticky pads, but probably the most important factor in reducing the load of dust and microorganisms is just regular cleaning with water. Ironically, high air flow in the laboratory, often used to remove toxicants, can exacerbate contamination by moving these microorganisms constantly throughout the laboratory. Furthermore, attention to sterile technique is important in reducing the amount of microorganisms in cultures.

Whether treatment of patients with antibiotics or washing of the vagina with iodoform will reduce potential contaminations during follicular aspiration has not yet been investigated. These potential solutions would still not address the possible microorganisms that may inhabit the ovary due to past aspirations or ascending infections.

1.19 How to Handle a Contamination Event

Notes:

What must be done when a contamination event occurs? The most important step to help identify a contaminant is to have a protocol in place well before the contamination occurs. Each clinic should have a written procedure on how to handle contamination. This protocol should address what and how to quarantine items, how to test items, and how to convey the event to the patient. One must communicate the event honestly and effectively to the physician and the patient, remove any potential contamination sources from use, and then identify why the contamination occurred.

1.20 Communication of the Contamination Event

Notes:

Communicating a serious event such as contamination takes a lot of skill to ensure that the information is accurate and does not lead the patient into misunderstanding why their embryos were affected. An open and honest policy is always best; the method of releasing this information should be handled by the physician in conjunction with risk management. Damage control can best happen if the physician, who has hopefully developed a good relationship with the patient, is the one to relay that the contamination occurred. Later, the embryologist can be brought in if there are specific questions about the culturing of the embryos. This information should always be relayed to the physician and patient as soon as possible after the occurrence. Delay can be interpreted by the patient as a method to cover up the event. These discussions are often better face-to-face as opposed to over the phone.

1.21 Handling Contamination

Notes:

When one observes overt contamination in an embryo culture (namely, flocculence), it is important to identify the source of this contamination so that it can be eliminated. The first step though is to quarantine all media, supplements, and devices that could have led to the contamination. These items should be marked as potentially contaminated and should be stored in special areas to prevent subsequent use and to preserve their condition for analysis. It is important that protocols be developed that will maintain the trail of evidence and that will allow for alternative sources of supplies while the suspected ones are analyzed.

1.22 Potentially Contaminated Media

Notes:

It is important that the protocol provides a method to mark and isolate any contaminated sources so that they are not inadvertently used. This may mean placing a bright tape on them that clearly states “Do Not Use - Potentially Contaminated. DO NOT DISCARD.” This media/item should then be placed in an area that is designated for expired or contaminated media. Potentially contaminated media/item should be stored in a place separate from general-use media so that it is not inadvertently used or accidentally discarded. It would be useful to check the media log to identify where and when these same suspected lots were used (or are being used). If a patient's embryos are in the same lots of suspected media/devices, one may want to try to remove them, rinse them, and place into a different lot.

1.23 How to Find the Source of Contamination

How to Find the Source of Contamination

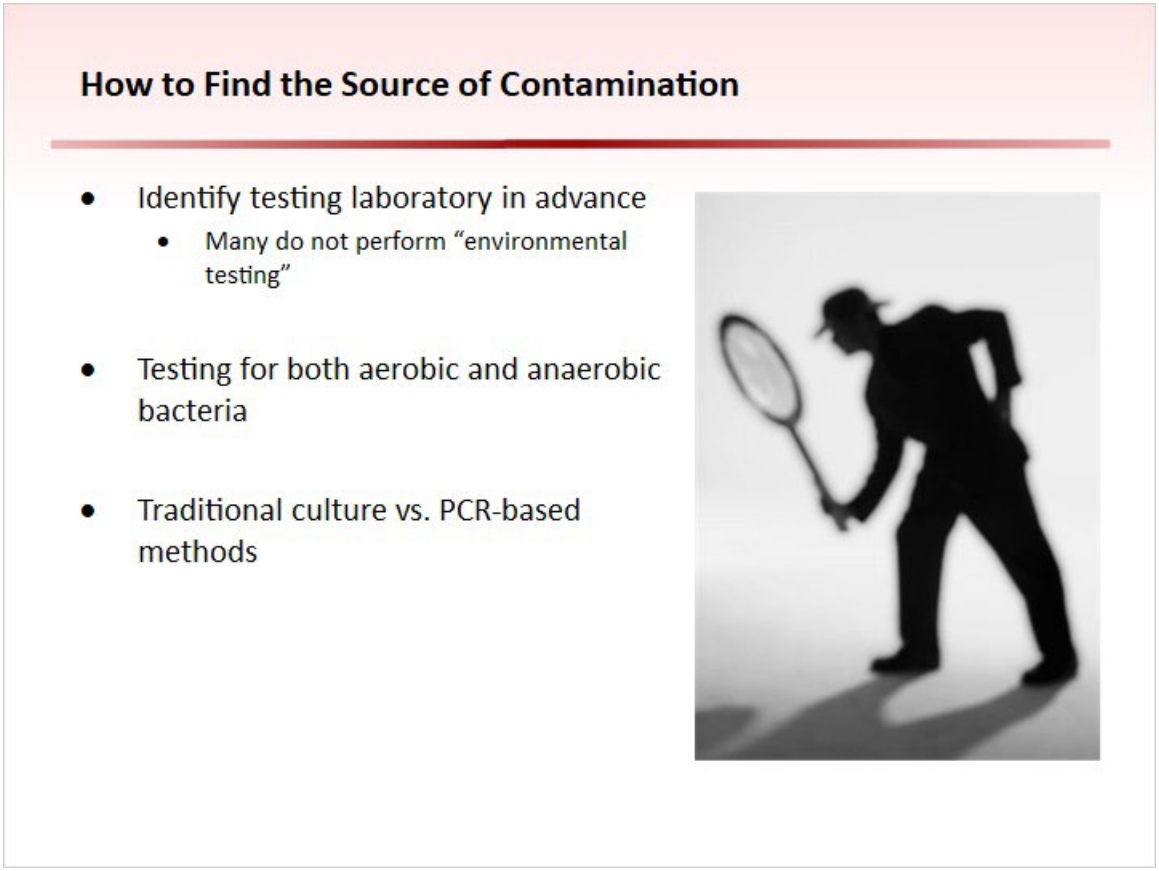
- Test the initial positive source: Media and supplements
- Primary sources
  - Semen
  - Follicular fluid
  - Empty cryogenic vial from any thawed tissue
  - Cryopreserved "sister" vials
- Secondary sources
  - Contact items
  - Sera
  - Media

Notes:

The first sources to analyze for contamination are often the media and supplements. Media that is obviously contaminated should be sent to a reference laboratory for identification of the organisms. Only a portion of the specimen should be sent so that some is reserved for future tests or in case the original is misplaced. One should try to rule out some sources (at least temporarily) by observing where contamination has occurred and where it has not occurred. For example, if contamination is only found in an embryo culture where embryos were placed, one could assume that this contamination had to come from follicular fluid, sperm, media used to work with follicular fluid (flush), or sperm (sperm preparation media). If drops without embryos are not contaminated, one could at least temporarily rule out culture media, oil, and the dish. If the same protein source was used in all of the media, it might be less likely a source if no contamination was noted in the embryo culture drops without embryos. Next, samples of media, follicular fluid, and semen should be sent to a reference laboratory to determine if any organisms are present. The latter is problematic as it is difficult to store fluid from each aspirate.
If a specific organism is identified, then any constituents of that medium should be examined for that contaminant. For example, if *Klebsiella* is identified in embryo culture

medium, then the serum used to supplement that medium should also be examined.

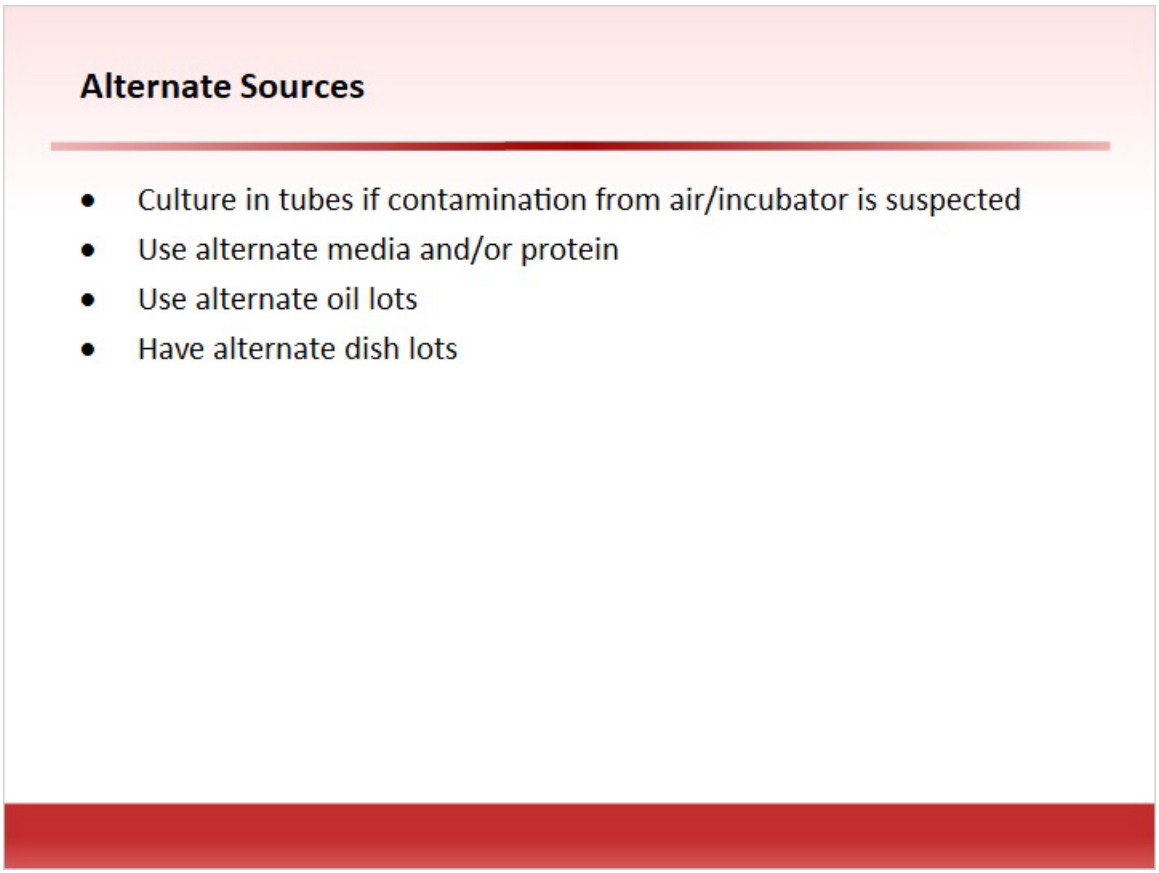
1.24 How to Find the Source of Contamination



Notes:

Testing laboratories should be identified well before any accident occurs in order to perform the testing as soon as possible after the event. Many of the traditional laboratories will not perform this "environmental" testing. Often, an environmental laboratory must be found or special arrangements made with a local reference laboratory. The type of testing will depend on where the contaminant is found. Although most obligate anaerobes can be excluded if growth occurred in culture media, the bacterium could be a facultative anaerobe. This may mean cultures should be for both types of bacteria, aerobic and anaerobic. Traditional culture methods using plates may be sufficient, but PCR-based methods are much more sensitive and should be used if available. If a culture method is being used, it will also be important to get the specimen to the testing laboratory in a timely manner so that the organisms do not die.

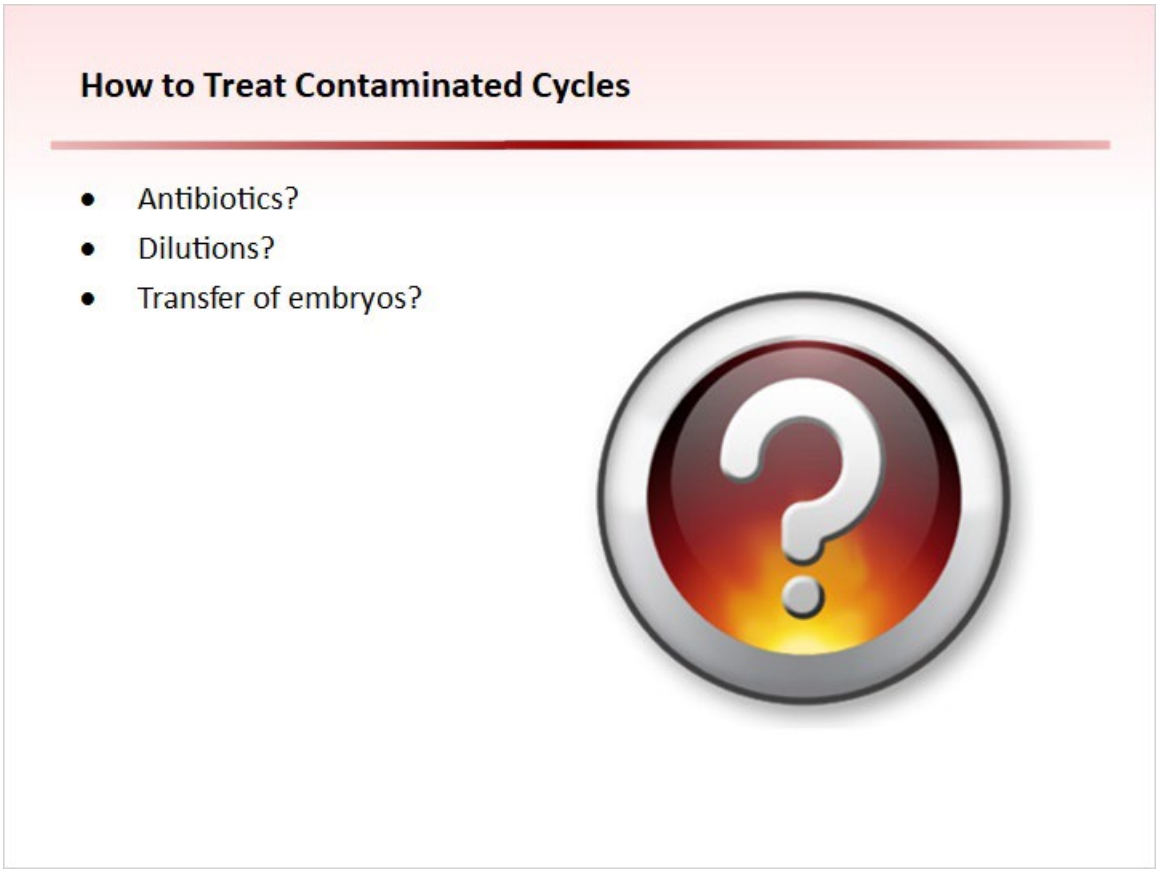
1.25 Alternate Sources



Notes:

None of the suspected contaminated sources should be used until a source is found. This necessitates that each laboratory has alternative sources to use during the investigation.
While the media/items are being tested, it is important that alternate sources be available for upcoming IVF cases. This can be handled by always having more than one lot of all media and contact materials on hand. Alternatively, materials may be shipped overnight or might be obtained from a nearby fertility clinic.

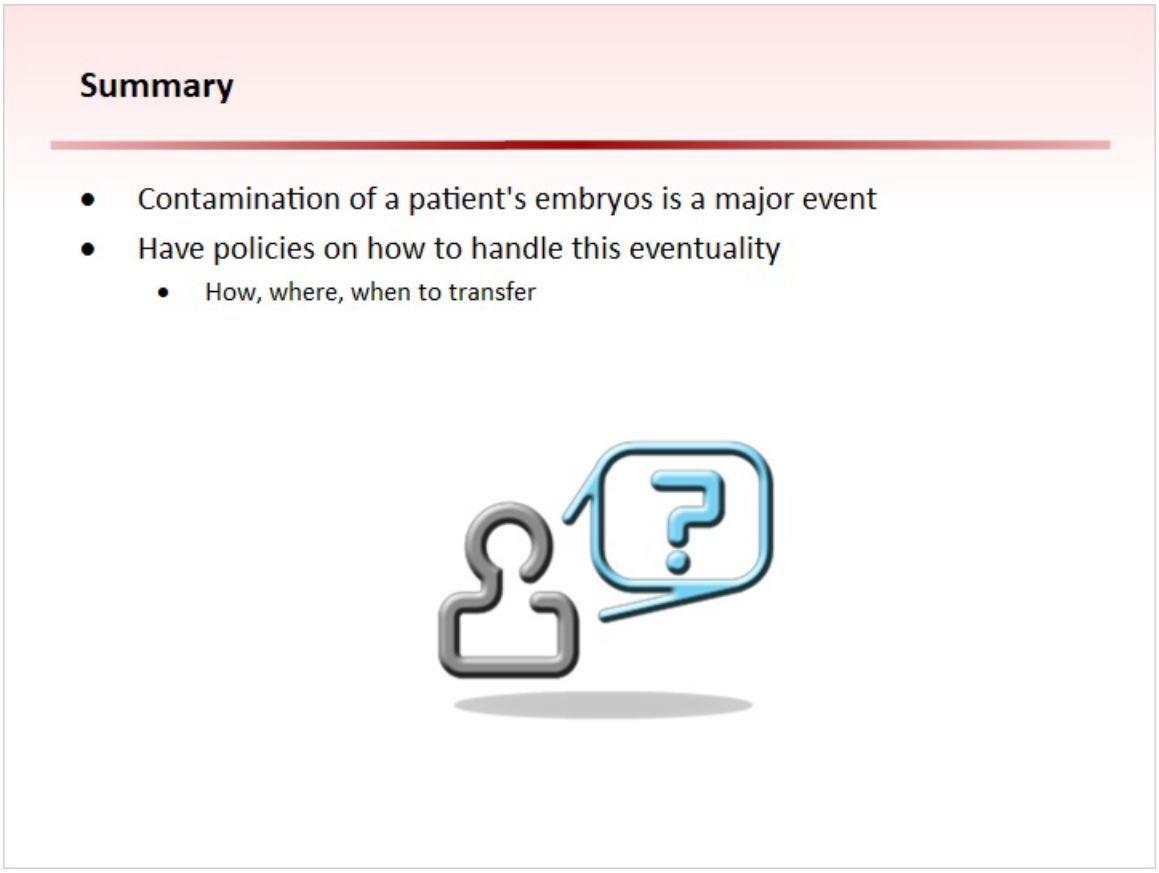
1.26 How to Treat Contaminated Cycles



Notes:

Treatment of contamination cycles is problematic. One must always ask if the various costs of the cycles are worth transferring a contaminated embryo and then endangering the health of the recipient (usually the patient). There are several cases where embryos contaminated with fungi were rescued by serial rinses, subsequently transferred into patients, and resulted in babies. No one, though, understands the risks of this procedure. What to do with potentially contaminated embryos is a problem that should be discussed in detail prior to any potential for its occurrence. Whether to transfer the embryos and the conditions for a transfer should be delineated in a clinic's protocols.

1.27 Summary



Notes:

In summary, contamination of media by microorganisms is a major event for IVF clinics. The most probable source of the contaminants is follicular fluid or semen, but all other sources should be ruled out. It is possible that most embryos are exposed to microorganisms during culture and that some IVF variability may be attributed to these inadvertent cocultures. Clinics need policies on how to handle contamination events in place prior to performing IVF. These policies enumerate how to handle the potential contaminants, how to test, where to test, and under what circumstances embryos can be transferred, if any.

1.28 Thank you!

Notes:

Thank you for participating in this educational activity.