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A history of adventitious agent contamination and the current methods to detect and remove them from pharmaceutical products

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ABSTRACT

Preventing adventitious agents from contaminating pharmaceutical products has been an important goal of regulatory agencies and industry for decades. Contamination of these products does not only erode consumer trust but also can have potentially serious health consequences. There are a wide variety of adventitious agents that can contaminate many different classifications of products, with each combination requiring different techniques for prevention or detection of adventitious agent contamination. This review seeks to give a brief overview of adventitious agents that have contaminated released pharmaceutical products, explain the different products that are at risk of contamination, then describe the methods commonly used for the prevention and detection of adventitious agent contamination.

1. What are adventitious agents?

The World Health Organization defines an adventitious agent as "contaminating microorganisms of the cell culture or source materials […] that have been unintentionally introduced into the manufacturing process of a biological product." [1] This is a fairly broad definition and, as a result, the scope of what can be considered an adventitious agent is quite large. Adventitious agents are replicative agents such as eukaryotes, prokaryotes, or viruses and additionally prions. A contaminating toxin without the presence of a replicative agent would not be considered an adventitious agent. Adventitious agents do not need to be known human pathogens and many adventitious agents are pathogens of animals that are typically considered to be non-pathogenic to humans. To yield a product safe from adventitious agents, any adventitious agents that are present need to be inactivated or, if the product cannot undergo inactivation, preventative steps must be taken to ensure contamination does not take place [2].

The contamination of pharmaceutical products with adventitious agents can have many negative effects. There is the most direct effect of someone experiencing an illness or injury due to a contaminated product. Contamination can cause shortages of drugs or vaccines as contaminated products must be removed from the market, such as when the only rotavirus vaccine was removed from the market for two months due to the detection of an adventitious virus [3]. From a practical standpoint, pharmaceutical companies are unable to profit from a contaminated, unsafe product. Additionally, there is a need for public trust in pharmaceutical products, as previous instances of adventitious agent contamination have led to consumer distrust, as parents who question vaccine safety are less likely to vaccine their children [4]. There was even a refuted conspiracy theory that a polio vaccine contaminated with an adventitious virus caused the Human Immunodeficiency Virus (HIV) pandemic [5].

2. Products have been released with contaminating adventitious agents

There have been many instances where adventitious agents have been detected within pharmaceutical products. This section seeks to highlight some of the events that have been especially impactful or allow insight into how regulations have been developed. A summary of these contamination events is included in Fig. 1.

2.1. The detection of Simian Virus 40 in a polio vaccine

In 1955, the Salk inactivated polio vaccine (IPV) was adopted for use in the United States and was produced by Merck [6]. The IPV was produced by formalin inactivation of polio virus cultured in rhesus monkey kidney (RMK) cells. In 1959 Eddy et al. identified that lysates from RMK cells contained an agent that caused tumors to develop in hamsters [7].

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Abbreviations

AGMK	African green monkey kidney cells
ALV	Avian Leukosis Virus
AOF	animal origin free
BVDV	Bovine Viral Diarrhea Virus
cDNA	complementary DNA
cGMP	current good manufacturing practices
CHO	Chinese hamster ovary cells
CJD	Creutzfeldt-Jakob disease
CPE	cytopathic effect
EAV-0	Endogenous Avian Retrovirus
FBS	fetal bovine serum
FDA	Food and Drug Administration
GSK	GlaxoSmithKline
Hib	*Haemophilus influenzae* type b
HIV	Human Immunodeficiency Virus
IPV	Salk inactivated polio vaccine
MMR	Mumps Measles and Rubella vaccine
PCR	polymerase chain reaction
PCV1	porcine circovirus-1
PERT	product-enhanced reverse transcriptase
RMK	rhesus monkey kidney cells
SFM	serum-free media
SV40	Simian Virus number 40
TSE	Transmissible spongiform encephalopathies
YF	yellow fever

Based on this research, and in the process of development of an adenovirus vaccine, researchers at Merck identified a contaminating agent of IPV that caused cytopathic effects in African green monkey kidney (AGMK) cells and identified the agent as Simian Virus number 40 (SV40) [8,9]. By the time SV40 was discovered, up to 98 million Americans had received a potentially contaminated IPV vaccine, although studies estimated that only 10–30% of people vaccinated with IPV received a contaminated dose [10,11]. There was evidence that the formalin inactivation procedures of IPV were able to inactivate SV40, although not as efficiently as poliovirus, but Merck began using AGMK cells for the production of IPV since SV40 is not an endogenous contaminant of AGMK cells [8–10]. By 1961, the Unites States government required all polio vaccines to be free of SV40, although there was no official recall of the contaminated doses of IPV [10]. Further studies have identified that rhesus macaques are natural carriers of SV40, so in the process of production of IPV the adventitious virus had been present for the entirety of culture in the RMK cells.

The impact of this contamination has been highly studied. SV40 is a polyomavirus and is related to the human BK and JC viruses [12]. In the laboratory, SV40 causes tumors and is able to immortalize cell lines with its T-antigen proteins, causing the primary concern regarding the contamination of IPV to cause recipients of the vaccine developing cancer [7,13,14]. SV40 antigens, as well as DNA, have been isolated from a variety of human cancers identifying these laboratory findings as potentially clinically relevant [12]. Despite the evidence that SV40 has the capability to cause cancer, many reviews of cancer rates following IPV vaccination have not found a connection between receiving a contaminated vaccine and cancer. Strickler et al. performed a retrospective cohort study focusing on the risk of SV40 associated cancers, comparing individuals that were exposed to contaminated IPV to those that were not based on age and found that exposure to the contaminated vaccine was not associated with increased rates of cancers [15]. A study of 700,000 individuals in Sweden that measured the incidence of osteosarcoma, brain ependymoma, and pleural mesotheliomas found there was no increase in incidence in individuals that were exposed to the contaminated IPV [16]. A 2003 Immunization Safety Review published by the Institute of medicine of the National Academies concluded that it was not clear if the presence of SV40 in the IPV directly caused cancer in any vaccine recipients [11]. There is, however, evidence that people who received IPV have seroconverted for SV40, but it is not clear if this is due to infection with the virus or exposure to formalin inactivated viral antigens present in the vaccine [10,17].

The contamination of IPV with SV40 was one of the first instances of identifying that a novel, endogenous animal virus can be carried from cell line preparation to production of a pharmaceutical product. It resulted in governmental regulators requiring screening for the specifically identified adventitious virus and the manufacturer changed the cell line used to continue production of the vaccine. Although the impact

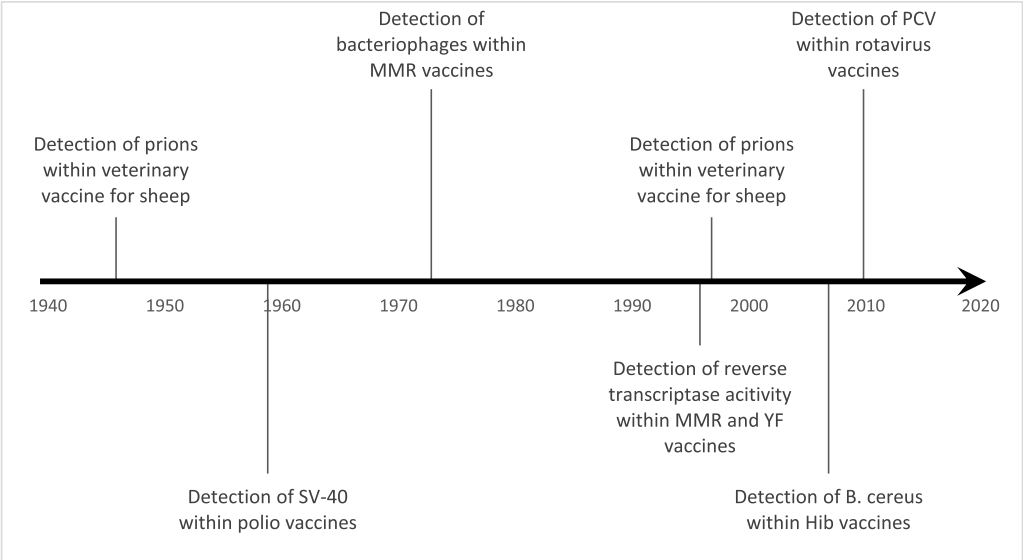

Fig. 1. A selected timeline of adventitious agent contamination
Each of the discussed events of adventitious agent contamination are listed chronologically, with the date in reference to the year the contamination was detected.

of the contamination is not fully understood, it does not appear to have had a measurable impact on the health of the tens of millions of people exposed.

2.2. The detection of phages in live attenuated vaccines

In 1973, the Food and Drug Administration (FDA) reported that bacteriophage, viruses that infect and replicate within bacteria, were present in multiple live attenuated vaccines, including the Mumps Measles and Rubella (MMR) vaccine and the oral polio vaccine [8,18]. This contamination was initially identified by unrelated studies on fetal bovine serum (FBS) and further studies went on to identify the contamination of vaccines that used the FBS for cell culture [19,20]. Initially, the FDA responded by forming a committee which decided to allow the contaminated vaccines to remain on the market until it could be determined if the phages could be removed from the vaccine production [8,18]. Studies into φV-1, a phage isolated from the MMR vaccine, did not detect effects of the phage on animal models or a persistence of the phage following inoculation [21,22]. Inoculation of nonhuman primates with φV-1 resulted in a robust serum antibody response despite the lack of viral persistence [22].

At the time, the collection of FBS was done in slaughterhouses where sterile techniques were not followed. Blood collected from the fetuses was routinely contaminated with bacteria and filtration was used to remove the bacteria [20]. This filtration was able to remove the comparatively large bacteria but allowed phages to contaminate the final FBS product. The use of this contaminated FBS in cell culture for live attenuated vaccines resulted in contamination of the vaccines given to people. Two years following the discovery of phages within the vaccines, the FDA moved forward with regulations requiring the FBS used in cell culture to be free of any contaminating phage, which caused the slaughterhouse industry to modify their practices.

The impact of the phage contamination of vaccines was not fully understood at the time. It is not known how many people were exposed to phage in contaminated vaccines, as live attenuated vaccines had been in use for over a decade at the time of this discovery. It was known that phages could transfer toxins to bacteria, causing them to be more pathogenic and there were also studies published that identified the ability for phages to deliver genes to cells in culture [20]. Now, although our vaccines are required to be free of phage, the biology of phages is better understood, and they are considered to be less dangerous to humans [23]. Recently, phages have been even approved for use as a compassionate use therapeutic against severe bacterial infections by the FDA [24]. Currently there is no evidence of any phage contamination directly causing harm to a human, and it is likely that the past contamination of vaccines did not directly cause adverse events.

2.3. Detection of reverse transcriptase within live attenuated vaccines

In 1995, both the live attenuated MMR and live attenuated yellow fever (YF) vaccines were cultured in chicken embryonated fibroblasts. At the time, it was known that avian retroviruses were a risk within eggs, and the FDA had a testing requirement for the detection of avian retroviruses within vaccines [8,25,26]. A method developed by Prya et al. in 1994 was able to detect reverse transcriptase activity with a 10^6 fold increase in sensitivity called a PERT assay [27]. The PERT assay was used to test vaccines and it was able to detect the presence of reverse transcriptase that was not detected by conventional methods [28]. Specifically, the MMR and YF live attenuated vaccines were found to be positive for reverse transcriptase activity, while inactivated vaccines cultured in chicken cells and eggs, such as the influenza vaccine, was not found to have reverse transcriptase activity. The reverse transcriptase was associated with the chicken embryonated fibroblasts, which are primary cells that are harvested from flocks of chickens. Further investigation identified that the reverse transcriptase activity was associated with the presence of Endogenous Avian Retrovirus (EAV-0) and Avian Leukosis Virus (ALV) nucleic acid and particles [29,30]. Despite detection of the retroviral nucleic acid, further studies were not able to isolate infectious virus from the chicken embryonated fibroblasts [26]. Tests of vaccine recipients showed no integration of provirus into peripheral blood lymphocytes as well as no seroconversion for ALV [31].

In response to the detection of a retroviral associated reverse transcriptase, the live attenuated vaccines were not removed from the market [8,26]. A WHO committee stated that "the risk of vaccine-preventable disease is real and quantifiable, whereas the risk posed by the chicken cell derived particles is theoretical and remote." [26] The general conclusion was that the PERT assay was able to detect the presence of non-infectious retroviruses that were endogenous to the chicken embryonated fibroblasts. Additionally, most people are exposed to avian retroviruses as part of the food chain, and serum collected from healthy poultry workers have been shown to have serological activity against avian retroviruses [32]. The use of alternate cell lines, such as human cells, can avoid the introduction of these avian retroviral elements completely however the longstanding safety record of chicken-cell derived vaccines has allowed the continued use of products containing low levels of reverse transcriptase activity.

2.4. Contamination of ovine vaccines with Scrapie

Transmissible spongiform encephalopathies (TSE) are a disease caused by an infectious protein called a prion. Exposure to a prion in sheep can lead to Scrapie, a neurodegenerative disease [33]. There have been two recorded instances of a European veterinary vaccine leading to the spread of scrapie within vaccinated sheep [34]. One identified instance of scrapie contamination was reported in 1946 by Gordon [35]. A louping-ill vaccine, composed of formalin inactivated infected sheep tissue, was released in 1934 to protect sheep from a tickborne flavivirus. Two years following vaccination, flocks of sheep in Britain began to display the symptoms of scrapie and Gordon went on to conclude that several sheep included in the vaccine preparation were in the early stages of scrapie but had not presented with symptoms. At the time, the causative agent of scrapie was not currently known, and Gordon was able to use the contaminated vaccine to identify that the infective agent of scrapie was within the brain and spine of affected sheep.

A similar situation was encountered in Italy in 1997 when the causative agent of Scrapie was understood. A vaccine was developed to protect sheep from contagious agalactia, a disease of sheep and goats that effects milk production and is caused by infection with *Mycoplasma agalactiae* [36]. The vaccine was produced by experimentally infecting sheep with *M. agalactiae*, harvesting infected tissue then inactivating the bacteria with formalin [37]. For both vaccines contaminated with scrapie, the contamination was only detected by observing illness in the animals that received the vaccine, after which vaccines were no longer administered. A retrospective cohort study identified that the provinces in Italy that received the contaminated vaccine had an increased rate of scrapie likely due to the presence of secondary transmission of the disease [37]. These instances of scrapie contamination highlight the importance of detection of contaminating prions within pharmaceutical products.

2.5. The detection of Bacillus cereus in a Hib vaccine

In 2007, there was a recall of over 1 million doses of a *Haemophilus influenzae* type b (Hib) vaccine due to the detection of *Bacillus cereus* in the manufacturing equipment [38]. This recall resulted in a shortage of Hib vaccines, leading to altered vaccine schedule recommendations [39,40]. The Hib vaccine was manufactured by Merck as PedvaxHIB and Comvax [41]. PedvaxHIB is a formulation of Hib capsule that is linked to the outer membrane proteins of *Neisseria meningitidis* for increased antigenicity. Comvax is a formulation of PedvaxHIB that also includes Hepatitis B antigen. PedvaxHIB is produced by the culture of Hib and *N. meningitidis* in fermentation media followed by purification of the

desired antigens. *B. cereus*, a soil bacteria, was detected in the manufacturing equipment, but there was not detection of any contamination within the final vaccine products [38]. Additionally, a study by Huang et al. tracked the reports of vaccine adverse events and did not find any increase in adverse events caused by the recalled lots compared to non-recalled lots released by Merck [42].

The potential *B. cereus* contamination of the Hib vaccine lots was not due to a contaminated animal product, but instead the lack of sterilization of equipment. It is an example of how there are many avenues for adventitious agents to enter pharmaceutical products.

2.6. PCV1 contamination of rotavirus vaccines

In 2004 and 2005, two rotavirus vaccines, Rotarix manufactured by GlaxoSmithKline (GSK) and RotaTeq manufactured by Merck, were approved for use [43]. The vaccines are live attenuated, with the virus serially passaged in AGMK and Vero cells prior to lyophilization [44]. Both vaccines were delivered orally to children worldwide.

A study by Victoria et al. in 2010 used next generation sequencing (NGS) to investigate the makeup of a number of vaccines, including Rotarix [45]. The sequencing of Rotarix did not identify any minority viral variants within the vaccine, however there were sequence reads that were attributed to porcine circovirus-1 (PCV1). The presence of PCV1 within Rotarix was confirmed by polymerase chain reaction (PCR) and microarray [45–47]. Following the publication of this research GSK performed additional investigation on the materials used to make the vaccines [48]. PCV1 DNA was detected at each stage of the production process, including the initial Vero cell banks that were used for the culture of the rotavirus were responsible for the contamination. In addition to detecting PCV1 DNA, there was infectious PCV1 detected in the rotavirus harvest and the purified bulk, but not the final filled vials. The final contamination of the vaccine is thought to have been carried from the Vero cell bank, with the cell bank being contaminated by porcine produced trypsin, since the trypsin used when the cell bank was initially produced in 1983 was not routinely irradiated [8,48].

Based on the findings of contamination of the GSK vaccine, Merck further investigated their stocks of RotaTeq. Merck identified the presence of both PCV1 and PCV2 DNA in the bulk lots and final vaccines of RotaTeq [8,49]. There was only DNA fragments identified; no infectious virus was recovered in either the bulk lots or the final vaccines [47]. Merck concluded that the PCV DNA came from the irradiated trypsin that was used in the culture of the Vero cells that were used for the culture of the vaccine [49].

At the time of the detection of PCV1 within the vaccines, over 100 million doses of Rotarix or RotaTeq had been administered worldwide, leading to serious safety concerns [50]. PCV1 is common virus of pigs that was first reported in 1974 and is not associated with any veterinary diseases [51]. PCV2 was first isolated in 1998 and is associated with wasting syndrome in pigs [51,52]. Both PCV1 and PCV2 are known as common contaminates of cell culture but neither PCV1 nor PCV2 are known to cause any disease in humans. Despite this, the FDA temporarily removed both Rotarix and RotaTeq, the only available rotavirus vaccines, from the market following the discovery of the PCV contamination for approximately two months [3,53]. The FDA allowed usage of the vaccines to resume and updated the labeling to include the presence of PCV1 since both vaccines had a strong safety records, there was no evidence that PCV causes disease in humans, and that the benefits of the vaccine are substantial [53]. Studies of vaccinated children identified that PCV was shed within the stool of some children following vaccination but vaccination did not lead to seroconversion for PCV1 [48,54]. Both Rotarix and RotaTeq are still used with PCV1 listed as an ingredient.

3. Types of products susceptible to adventitious agent contamination

All manufactured products are at some risk of adventitious agent contamination from environmental, laboratory, or operator-based contamination. Along with the universal risk of this type of contamination, some manufacturing methods are at substantially higher risk of adventitious agent contamination, resulting in some types of products being more prone to contamination. Products produced in cell culture, products that require animal-based reagents for production and products that require the direct use of animals are at the highest risk of adventitious agent contamination. While many products are produced using these methods, vaccines, recombinant proteins, and monoclonal antibodies are products that see wide-spread use and are commonly manufactured using these methods.

Vaccines, recombinant proteins, and monoclonal antibodies that are produced without the use of cell culture are at a lower risk of adventitious agent contamination. Recombinant proteins can be produced by fermenting yeast, which considered to be fairly low risk for contamination with adventitious agents since yeast cannot support pathogens [55,56]. There is currently research into the use of fermentation techniques to produce complex human proteins, such as monoclonal antibodies, which in the future many be implemented do decrease the risk of contamination [57,58].

Vaccines are produced in a variety of ways with varying risk of adventurous agent contamination. Some vaccines are produced by culture of bacteria, which, like yeast fermentation, has a low risk of adventitious virus contamination. Generally, live attenuated vaccines tend to have a higher risk of adventitious agent contamination than inactivated vaccines, as the steps of inactivation can also render the adventitious agent non-infectious. There are, however, viruses and prions that are resistant to formalin inactivation so there is still a risk of contamination in inactivated products [35,37,59].

Live cell products, such as Chimeric Antigen Receptor T-cells, are also at high risk of adventurous agent contamination due to the stability requirements of final material [60]. Since the cells need to be administered live it is difficult to remove any adventurous agents that may be present because most purification methods will damage the desired cells. An additional complication to live cell products is that they must be collected from a host, leading to an additional potential source of adventitious agent contamination.

4. Sources of adventitious agent contamination

4.1. Use of cell lines with endogenous adventitious agents

All cell lines were at some point harvested from a host and some cells that were used to develop these cell lines contained endogenous adventitious agents at the time of collection. The use of these cell lines in pharmaceutical product development can lead to adventitious agents contaminating the final product. The adventitious agents introduced in this way tend to be native to the species that the cells were collected from. For example, RMK cells naturally contain SV-40, a variety of insect cell lines that are used for recombinant protein expression naturally harbor Sf-rhabdovirus, and a mosquito cell line used for the production of arboviruses was shown to be contaminated with a mosquito parvovirus [7,9,61,62]. Primary cells, such as chicken embryonated fibroblasts used for the production of the MMR vaccine that contains EAV-0 and ALV, are also a potential source of adventitious agent contamination [29,30].

4.2. The use of animal products in cell culture

Cell culture requires complex materials, such as growth factors, essential amino acids, and enzymes, that are harvested from animals due to the cost or difficulty of production. The most commonly used animal

products in cell culture are FBS, which is collected from fetal cows, and trypsin, an enzyme collected from the pancreas of pigs. FBS has been associated with contamination with bovine viruses such as bovine viral diarrhea virus, bovine parvovirus, ungulate tetraparvovirus 2, and bovine hepacivirus [63–65]. Poor collection methods of FBS have also resulted in bacterial contamination as well as phage contamination [19,20]. Trypsin has been found to contain a number of different viruses of porcine origin, such as PCV1, and classical swine fever virus [63]. Changes to collection techniques of animal products, as well as treatment, such as filtration and irradiation, prior to use have reduced the risk of adventitious agent contamination [66].

4.3. The use of animals for direct production of a product

Some products do not use *in vitro* cell culture but instead rely directly on animals, or animal products, to produce pharmaceutical products. Endogenous infectious agents of these animals then can contaminate the final pharmaceutical product. A common example of this is the use of eggs for the culture of viruses for use in vaccines. Avian retroviruses have been detected in products cultured in eggs [28]. Another use of animals is the culture of microorganisms through experimental infection. This can lead to the transmission of any other microorganisms the animal was infected with as well as any prions that might be present in the animal. The methods of smallpox vaccine production in the 1950s to the 1980s involved the collection of vaccinia virus infected animal skin, which put the product at a high risk of adventitious agent contamination [67].

4.4. Environmental contamination

The laboratory environment can also be a source of adventitious agents. Bacteria are present on most surfaces if they are not disinfected.

Table 1
A selection of potential adventitious agents.

Name	Abbreviation	Classification	Source of adventitious agent	Ref
Bacillus anthracis		Bacteria	Animal skin	[67]
Bacillus spp.		Bacteria	Environmental contaminant	[67]
Brucella spp		Bacteria	Animal skin	[67]
Clostridium spp.		Bacteria	Animal skin	[67]
Micobacterium spp.		Bacteria	Environmental contaminant/animal skin	[67]
Mycoplasma spp.		Bacteria	Environmental contaminant	[121,122]
Aspergillus spp.		Fungus	Environmental contaminant	[123]
Candida spp.		Fungus	Environmental contaminant	[123]
Bovine Spongiform Encephalopathy	BSE	Prion	Nervous tissue of Cows	[67]
Chronic Wasting Disease	CWD	Prion	Nervous tissue of Elk	[124]
Creutzfeldt-Jakob disease	CJD	Prion	Nervous tissue of humans	[124]
Kuru		Prion	Nervous tissue of humans	[124]
Scrapie		Prion	Nervous tissue of Sheep	[124]
Avian retroviral particles		Retro-viral like particles	Eggs or avian primary cell lines	[45,125]
Endogenous retroviral particles		Retro-viral like particles	Various cell lines	[8]
Adenovirus		Virus	Humans and a contaminant of gene therapy	[122,126]
Avian leukosis virus	ALV	Virus	Eggs or avian primary cell lines	[122]
BK virus	BKV	Virus	Human products/human contact	[122]
Blue Tongue Virus	BTV	Virus	Bovine and ovine products	[126]
Bovine Parainfluenza virus Type 3		Virus	Bovine products	[67]
Bovine viral diarrhea virus	BVDV	Virus	Bovine products	[67]
Cache Valley Virus	CVV	Virus	Ovine products	[126]
Cytomegalovirus	CMV	Virus	Human products/human contact	[67]
Epstein–Barr virus	EBV	Virus	Human products/human contact	[67]
Hepatitis A	HAV	Virus	Human products/human contact	[67]
Hepatitis B	HBV	Virus	Human products/human contact	[67]
Hepatitis C	HCV	Virus	Human products/human contact	[67]
Herpes B virus		Virus	Non-human primate products	[126]
Human enteroviruses		Virus	Human products/human contact	[67,89,122]
Human Herpes virus 6, 7, 8	HHV6, 7, 8	Virus	Human products/human contact	[126]
Human immunodeficiency virus	HIV	Virus	Human products/human contact	[67]
Human papilloma virus	HPV	Virus	Human products/human contact	[67]
Human parvovirus		Virus	Human products/human contact	[127]
Human T-lymphotropic virus	HTLV	Virus	Human products/human contact	[67]
Influenza		Virus	Human products/human contact	[67,89]
JC virus	JCV	Virus	Human products/human contact	[122]
Measles		Virus	Human products/human contact	[89,122]
Minute virus of mice	MVM	Virus	Murine contamination	[122,126,128]
Mouse parvovirus	MPV	Virus	Murine contamination	[128]
Norovirus		Virus	Human products/human contact	[67]
Parainfluenza virus		Virus	Human products/human contact	[126]
Porcine circovirus	PCV	Virus	Porcine derived trypsin	[126]
Respiratory syncytial virus	RSV	Virus	Human products/human contact	[67]
Rubella		Virus	Human products/human contact	[89,122]
Sf-rhabdovirus		Virus	Insect derived cell lines	[61,129]
Simian Virus 40	SV40	Virus	Non-human primate cells	[122]
Vesicular Stomatitis Virus	VSV	Virus	Products of insects, cattle, horses, or pigs, as well as contamination of transgenic cells	[89]
Avian polyomavirus		Virus	Eggs or avian primary cell lines	[122]
Simian Cytomegalovirus		Virus	Non-human primate cells	[130]

Additionally, laboratory personnel can be a source of adventitious agents, as microorganisms that originate from a person can often replicate within cell culture. One common example of environmental contamination is mycoplasma, a bacterial contaminant that often originates from laboratory personnel [68]. Proper sterile technique at all stages of production is required to prevent environmental contaminating agents from entering pharmaceutical products.

5. Common contaminating agents

There are a wide variety of agents that can act as adventitious agents. Below, different classifications of contaminating agents are discussed, as well as their potential impact on pharmaceutical products. A list of selected adventitious agents is presented in Table 1.

5.1. Bacterial contaminants

Cell cultures can often become contaminated with bacteria as cell culture medium contains the necessary nutrients for the growth of bacteria. Bacteria are common in the environment as well as on laboratory workers and bacteria from the environment can contaminate cell cultures or products used in cell culture if sterile conditions are not maintained. There are many different types of bacteria that can cause contamination [69]. Often contaminating bacteria will be visible by microscopy, visible as cloudy supernatant macroscopically, or are detectable by a change in the pH indicator of the media [70].

A very common bacterial contaminant of cell cultures are *Mycoplasma* spp. Studies of thousands of cell lines identified that about 25% of cell lines were contaminated by mycoplasma [68]. *Mycoplasma* spp. are gram negative bacteria that are small in genome length as well as size, with most *Mycoplasma* having a genome less than 1 Mb long and are a spherical shape that is 1–2 μm in length and 0.1–0.2 μm wide [71,72]. The small size of *Mycoplasma* spp. allows them to pass through 0.2 μm filters. Often Mycoplasma contamination cannot be detected by microscopy as the bacteria are too small to observe causing molecular techniques to be required. There are many commercially available PCR assays for the detection of Mycoplasma within cell culture [73].

Bacterial contamination of pharmaceutical products can cause harmful effects in people who receive the products [74]. Contamination of pharmaceutical products with pathogenic bacteria can directly lead to infection. Even if the bacteria are killed by inactivation methods, toxins produced by the bacteria can still be active, leading to adverse effects.

5.2. Eukaryotic contaminants

Like bacterial contaminants, there are also eukaryotic organisms in the environment that can replicate within cell culture conditions. As cell culture conditions are designed for the replication of eukaryotic cell lines, adventitious eukaryotic agents can often replicate as well. Additionally, while many types of bacterial contamination can be prevented by the addition of antibiotics to the media, most compounds that would kill eukaryotic contaminants would also kill the cells of interest within the culture, although there are available anti-fungal compounds that can be used in cell culture conditions [75]. The most common eukaryotic contaminants are fungi but parasites such as single-celled protozoa can also contaminate cell cultures [69,70]. Like bacteria, eukaryotic contamination often originates from the environment and contamination is often detectable by microscopic examination due to the large size of eukaryotic organisms.

Contamination of pharmaceuticals with eukaryotic organisms can lead to serious disease. For example, a lot of methylprednisolone acetate that was contaminated with fungi lead to fungal meningitis in patients that received the drugs [76].

5.3. Viral contaminants

Unlike bacterial and eukaryotic contaminants which can be found living in the environment, viral contaminants must originate from a host. While laboratory workers can be the source of a contaminating virus, most viruses enter a cell culture through another contaminated product; products isolated from animals are often the source of viral contamination [77]. Additionally, there are many cell lines with endogenous viruses that can cause contamination. Often, these endogenous viruses play a role in the immortalization of the cell lines and can be integrated to the cellular genome. Chinese hamster ovary (CHO) cells and murine hybridomas have been shown to contain endogenous retroviruses as well as virus-like nucleic acids [77,78].

Since contaminating viruses often originate from animal products used in cell culture, many common adventitious viruses originate from animal species commonly used in biomanufacturing. Bovine viruses are common due to the use of serum, porcine viruses are common due to the use of trypsin, and avian viruses are common due to the use of eggs in biomanufacturing techniques [29,63,79]. Viruses of humans and nonhuman primates are also common contaminants, often originating from the initial collection of the cell lines used for production [10]. There is also concern with viruses of human origin contaminating pharmaceutical products. A human associated virus can enter a cell culture either using products of human origin or through contamination by infected laboratory workers.

Retroviruses and retroviral elements require special consideration in cell cultures due to their unique biology. Retroviruses can exist as enveloped virions containing RNA or as a provirus that is DNA integrated into the host cell's genome. Retroviruses also have the risk of causing disease by integrating within important genes of the host genome causing even non-replicative retroviral agents possessing integrase to be potentially dangerous, although there have been no reported instances of this taking place.

People are regularly exposed to viruses in the environment and in their diets and most viruses have no effect on humans; only a small subset of known viruses are infectious to people. There is evidence that people exposed to many types of animal viruses through the food chain or through agricultural work without any illness [32,54]. Despite this fact, the infectious potential of most viruses is not known in humans, causing an abundance of caution when evaluating viral contamination of pharmaceutical products.

5.4. Prion contaminants

Prions are infectious, non-living agents that are composed of entirely of protein. Most mammals produce a protein called prion protein (PrP), which can be folded in two structural variants: PrP^{C} and PrP^{Sc}. PrP^{C} is present in healthy individuals while PrP^{Sc} is present in those with a prion disease [80]. When PrP^{C} is exposed to the PrP^{Sc} isoform of the protein the PrP^{C} can change to the highly stable PrP^{Sc} isoform. As PrP^{Sc} accumulates within the brain, neurologic diseases called TSE can develop. PrP^{Sc} is highly resistant to heat, detergents, formalin, and autoclaving making inactivation of the infectious protein difficult [59,81]. Prions are found in the nervous tissue of effected animals so there is a risk of contamination whenever animal tissue is used in a pharmaceutical product. People exposed to prions are at risk for TSE such as kuru, Creutzfeldt-Jakob disease (CJD), variant CJD, fatal familial insomnia and Gerstmann–Sträussler–Scheinker, and these diseases are responsible for up to 300 deaths annually in the United States [80,82].

6. Methods for the detection of adventitious agents

Due to the range of adventitious agents that can contaminate products, there are many types of assays that are commonly used for the detection of adventitious agents. These assays are described below, along with situations that they are commonly used. A summary of these

assays is presented in Table 2.

6.1. Visual and microscopic inspection

Although they are small, it is possible to observe growth of microorganisms with either visually or with a microscope. Contamination of cell cultures with bacteria or fungi can lead to visible growth within the culture flasks and cloudy media can often be a sign of contamination. The use of visual inspection is routinely performed during cell culture processes, so it is an inexpensive and quick method for the detection of adventitious agents. Not all adventitious agents are visible by microscopy; viruses and even some bacteria cannot be visually observed without targeted staining or the use of an electron microscope. Despite being unable to detect many types of adventitious agents, microscopic screening is still a useful tool for the detection of adventitious agents.

6.2. Detection of cytopathic effect in culture

Although viruses are too small to directly detect visually, it is possible to observe the effects of viral replication on cells. Often, cells infected with a virus will display some form of cytopathic effect (CPE), a morphological change to the cells that is caused by the virus [77,83]. For a typical *in vitro* CPE assay, a variety of cell lines are set in flasks then each challenged with the sample that is to be tested. After this challenge, the cells are observed, and potentially sub cultured, over approximately a month for the appearance of any CPE [84]. Common examples of CPE include the formation of syncytia (fusion of cells resulting in a single cell with many nuclei), change of cell shape, the development of inclusion bodies, rounding of cells, clumping of cells, and total or subtotal destruction [85,86].

The advantages to screening for CPE to detect adventitious viruses are that the procedure is relatively simple and inexpensive to perform, and it is not targeted to specific viruses as a wide variety of viruses can cause some form of CPE. The main drawback to using CPE assays is that some adventitious viruses only cause CPE in specific cell lines that might not be used in the assay and other viruses do not cause any CPE when replicating within cells. Berting et al. reported that a variety of important adventitious viruses did not cause CPE in CHO cells including Bovine viral diarrhea virus, Coxsackievirus B3, Murine hepatitis virus, Pseudorabies virus, Porcine parvovirus, and Parainfluenza Virus 3 [83]. While most of these viruses were not able to replicate within CHO cells, Parainfluenza Virus 3 was able to modestly replicate within CHO cells while not producing any CPE. However, if mouse astrocytes are used instead of CHO cells, Coxsackievirus B3 is able to replicate and cause CPE such as rounding of cells, cytoplasmic blebbing, and detachment from the monolayer indicating the need for testing with proper cell lines [87]. When screening live attenuated viral vaccines, it can be difficult to distinguish CPE caused by the viral component of the vaccine or by a contaminating agent, so consideration of the product that is being tested is also necessary. Additionally, a CPE assay is only able to screen for the presence of viruses, not adventitious agents of other categories.

An *in vitro* assay that is similar to the detection of CPE is a hemagglutination assay. Many viruses possess proteins that attach to the surface of red blood cell and a suspension of viruses with these proteins has the ability to cause red blood cells to clump together, or agglutinate [88]. In a hemagglutination assay, cells are infected with virus then red blood cells are added to the suspension. If the added red blood cells agglutinate, then the culture is considered to be hemagglutination positive, so there is considered to be virus in the well. This assay has the ability to detect viral replication in the absence of CPE, but it requires culture of an adventitious virus in a susceptible cell line. Additionally, not all viruses are capable of hemagglutination, so this assay is not able to detect all classifications of adventitious viruses.

6.3. Injection into animals

Since it has been known that adventitious agents can only be cultured in highly specific *in vitro* conditions, *in vivo* animal testing has been used to screen for the presence of adventitious agents. The main advantages of *in vivo* testing are that the tests are completely non-targeted, and any classification of adventitious agent should be able to cause disease within animals; animal testing is one of the few methods able to detect prion contamination. The main disadvantages of animal testing include the high cost and amount of time required to observe the animals. Not all adventitious agents are able to cause disease in laboratory animals. Many dangerous human pathogens, such as HIV, are unable to cause disease within animals due to the highly specific nature of the agents' pathogenesis. Additionally, it is a general goal of science to reduce and replace the use of animals, so the use of dozens of animals for screening is not ethically ideal.

The FDA has put forward recommendations for the use of animals in adventitious virus testing [2]. Adult mice, suckling mice, guinea pigs, rabbits and embryonated chicken eggs are recommended for use. Each of these animals is recommended for the detection of different types of adventitious agents. For adult mice, at least 20 mice should be inoculated intraperitoneally and intracerebrally with the product and

Table 2
A selection of assays for the detection of adventitious agents.

Assay name	Assay classification	Assay target	Agents detected	Use of the assay	Citations
Visual inspection	Inspection	Biofilms or filamentous growth	Bacterial or Eukaryotic	Any contamination with a large organism can be visually observed.	
Microscopic inspection	Inspection	Cells	Bacterial or Eukaryotic	Material is examined under a microscope. This examination can include differential or targeted staining.	
Cytopathic Effect	Culture	Infectious virus	Viral	Material is used to inoculate cell culture, and effects on the cellular monolayer are observed.	[77, 83–86]
Hemagglutination assay	Culture	Infectious virus	Viral	Material is used to inoculate cell culture, and supernatant of this culture is tested for its ability to bind to red blood cells.	[88]
Infection of animals	*in vivo* challenge	Infectious material	All	Material is inoculated into animals and death or sickness of the animal is recorded.	[2,89]
Infection of eggs	*in vivo* challenge	Infectious material	All	Material is inoculated into embryonated eggs and embryos are examined and egg contents are tested for hemagglutination.	[2]
Antibody production test	*in vivo* challenge	Antigens of known pathogens	Viral, Bacterial or Eukaryotic	Material is inoculated into animals, serum is collected and tested for binding to known contaminants.	[2]
Product-enhanced reverse transcriptase assay	Molecular	Reverse Transcriptase	Retroviral	RNA is added to the material, then the material tested for the presence of cDNA.	[27,28, 90]
Polymerase Chain Reaction	Molecular	Nucleic Acid	Viral, Bacterial or Eukaryotic	Nucleic acid is isolated from the material, and tested for the presence of known sequences.	[92–94]
Next Generation Sequencing	Molecular	Nucleic Acid	Viral, Bacterial or Eukaryotic	Nucleic acid is isolated from the material, and sequenced.	[45,97]

monitored for at least 21 days. If less than 80% of the mice remain healthy than the product cannot be used. Suckling mice are used similarly to adult mice except that suckling mice should be observed for 14 days and tissues from surviving mice should be passaged into an additional 5 sucking mice which are also observed. For embryonated eggs, 10 eggs should be inoculated with the material then incubated for 3 days. After the incubation, the allantoic fluids should be collected then injected into a new set of eggs and incubated for an additional 3 days. The embryos should be examined after the incubation and the contents of the egg should be tested for hemagglutination, where 80% of the eggs should appear normal and be free of hemagglutinating agents. An additional screening of embryonated eggs should also be completed where 10 additional eggs are inoculated with the product then incubated for 9 days. The embryos are examined after the incubation and the product passes the test of at least 80% of the embryos appear to be normal. Generally, each animal that succumbs during this testing must be tested for the presence of adventitious agents within its tissues by secondary means.

Gombold et al. performed an evaluation of *in vivo* methods for the detection of adventitious viruses compared to *in vitro* methods [89]. Serially diluted viral stocks were used to inoculate adult mice, suckling mice and embryonated chicken eggs, and the dilution at which *in vivo* techniques were able to detect the virus was determined. Of the 11 adventitious viruses that were tested using *in vivo* methods, only six were detected. Interestingly, using a variety of *in vitro* techniques, all 11 of the adventitious viruses were detected, typically at much lower concentrations than were detectable by the *in vivo* assays. This study brings the sensitivity of the typical *in vivo* testing methods into question and highlights the need for potential alternate screening methods for adventitious agents.

An additional type of *in vivo* test that can be used is an antibody production test [2]. In this test, specific pathogen free animals are inoculated with the product that is being tested. Following inoculation, serum is collected from these animals then tested for the presence of antibodies against chosen adventitious agents. This method does not require the animals to be affected by the adventitious agent since an antibody response can take place without replication of the adventitious agent, but since the antibody screening is done in a targeted manner there is a limit to the number of adventitious agents that can be detected.

6.4. Detection of reverse transcriptase activity

The defining characteristic of a retrovirus is its ability to reverse transcribe its RNA genome to DNA for integration into a host genome. The protein responsible for this is called reverse transcriptase. Assays have been developed to detect the presence of reverse transcriptase by detecting the conversion of RNA to DNA. If reverse transcriptase activity is detected, then it is assumed that a retrovirus or a retroviral-like element is present within the sample. These assays are very sensitive and can detect low levels of retroviral contamination, however they are unable to detect other forms of adventitious agent contamination.

One assay that detects the presence of reverse transcriptase is the product-enhanced reverse transcriptase (PERT) assay [27,28]. In a PERT assay, bacteriophage RNA, and a complementary primer are added to the product and incubated. Any reverse transcriptase in the sample will transcribe the phage RNA to complementary DNA (cDNA). Following the incubation, a PCR is performed to amply any present cDNA. The initial RNA that was added to the product will not be amplified by this PCR step. Following PCR amplification, the presence of phage cDNA is then detected by Southern blot. As methods have developed, florescent-PERT (f-PERT) has been developed, which used qPCR for the detection of cDNA to increase the sensitivity [90].

6.5. Targeted detection of nucleic acid

PCR is a highly sensitive method for detecting the presence of adventitious agents. Most adventitious agents have a nucleic acid component to them that can be detected so PCR can be used for the detection of viruses, bacteria, as well as eukaryotic organisms. To detect an adventitious agent by PCR, first the nucleic acids must be extracted from the material being tested. Following extraction, targeted primers and enzymes are added then the sample is incubated in a thermal cycler. The design of the primers used can allow for detection of either a specific strain of an organism, or for detection of a nucleic acid sequence that is conserved within a family of organisms. Reverse transcriptase enzymes can be used to produce cDNA for PCR reactions, allowing for PCR assays to detect with DNA or RNA. DNA is typically detected by fluorescence produced by probe hydrolyzation during amplification, which allows for efficient DNA detection without the need to run a gel.

Theoretically, the method of PCR is able to detect as few as a single copy of the target nucleic acid. In practice, although not perfect, PCR is able to detect adventitious agent nucleic acid with an incredibly high sensitivity. Demonstrating the high sensitivity of PCR assays, Bae et al. developed PCR assays for the detection of minute virus of mice, bovine parvovirus and bovine herpesvirus with reported sensitivities of 6.49×10^1, 7.23×10^2 and 5.8×10^1 $TCID_{50}$/mL respectively [91]. Similarly, Oh et al. developed reverse transcriptase PCR assays for the detection of reovirus, bovine viral diarrhea virus, and bovine parainfluenza virus with reported sensitivity of 7.76×10^2, 7.44×10^1 and 6.55×10^1 TCID10/mL respectively [92].

PCR is routinely used for the detection of mycoplasma contamination of cell culture because culture-based methods can take up to two weeks to complete, and due to the size of the bacteria it is difficult to detect with microscopic methods. There are many strains of mycoplasma that can contaminate cell cultures, so design of the PCR assays must account for the potential differences in sequence between the different strains. For detection of mycoplasma, primers that target conserved regions of the genome, or multiple primer sets are used to ensure sensitive detection [73,93,94]. These PCR methods have been shown to be highly sensitive, with Sung et al. reporting that their developed PCR assay is able to detect 10 CFU/mL mycoplasma within cell culture medium [94].

Detecting nucleic acid of adventitious agents is highly sensitive, inexpensive and can be completed in under a day making it one of the preferred methods for the detection of adventitious agents. Recently, advancements of multiplexing have allowed for detection of multiple adventitious viruses in a single PCR reaction, allowing for increased speed of sample screening [63,91,92].

6.6. Next generation sequencing

Adventitious agent nucleic acid can be detected using next generation sequencing (NGS) technologies as well as by PCR. NGS technologies are currently being explored as a primary method for the detection of adventitious agents due to its low cost, quick runtime, and ability to detect a wide variety of agents. Since NGS does not require targeted primers it can detect previously unidentified viruses, as is often done in environmental screening studies, and a single NGS assay can be used in place of numerous PCR assays.

NGS refers to methods that have been developed for the sequencing of nucleic acid beyond the traditional Sanger sequencing methods such as sequencing by hybridization, sequencing by synthesis, nanopore sequencing, and long-read sequencing [95]. These methods have consistently improved for read length, number of reads, and run time. NGS methods typically involve preparation of the nucleic acid, sequencing of the nucleic acid, then bioinformatic analysis. There are many options that have been tested for library preparation, including targeting of either DNA or RNA, steps where adventitious agent nucleic acid can be enriched, or host nucleic acid can be depleted [78]. The sequencing can either be targeted using predetermined primers if there is a specific sequence that needs to be sequence, or non-targeted where all the nucleic acid present is sequenced. There are many sequencing platforms that can be used including Illumina, Oxford Nanopore

Technologies, PacBio, and Ion Torrent. The bioinformatic analysis can be modified based on if the sequencing is targeted, or if you are. Additionally, there are sequence databases that are being developed for the detection of adventitious viruses through bioinformatic analysis such as the Reference Viral Database [96].

One of the most noteworthy uses of NGS for the detection of adventitious agents was completed by Victoria et al. [45], where NGS technologies were able to detect the presence of an adventitious virus when the planned goal of the experiment was to assess the sequences of the vaccine. Victoria et al. [45] also performed similar sequencing techniques on a variety of live attenuated viral vaccines, and identified contamination with avian leukosis virus and simian retrovirus. Following this publication, many other groups have shown the ability of NGS techniques to detect adventitious viruses [97].

For the adoption of NGS platforms for the detection of adventitious agents there are hurdles than need to be overcome. In most products, any contaminating agents would be expected to be present at an extremely low level, especially in products that contain cellular products, and methods to exclude host reads, or to enrich for contaminants, may be required to ensure sensitivity of detection. An additional challenge to overcome is the time required for some sequencing technologies. Although sequencing can be completed substantially faster than traditional *in vivo* methods, some NGS methods can take multiple days for sequence and analysis which limits the usefulness to operators.

6.7. False positives when testing for adventitious agents

Due to the high sensitivity of the assays that are used to detect adventitious agents, false positives are a major concern to groups performing the tests. False positives can result in increased costs due to additional testing or increased regulatory hurdles so it is desirable to ensure a low false positivity rate. Nims et al. highlights that the tests themselves can become contaminated from testing equipment, the testing reagents, the test operator, or the environment [98]. There are a variety of reports of laboratory contamination of testing reagents that have led to false positives in many fields of study [99–102]. Similarly to the how a contaminated animal product can lead to the contamination of a pharmaceutical product, the use of a contaminated FBS can lead to false positives for serologic or molecular tests for BVDV [103]. As discussed above, products produced in cell culture are at a higher risk of adventitious agent contamination, and that trend continues to the production of laboratory reagents. Monoclonal antibodies used in testing procedures are often manufactured less stringently than the pharmaceutical products that are being tested, so false positives can occur.

Adventitious agent tests can also yield false positives in the absence of contamination. Talarico et al. describe a false positive testing event where the proteins included in an influenza vaccine were able to cause hemagglutination of mammalian red blood cells [104]. This finding took over five months to fully confirm, resulting in a delay of vaccine production that could have been avoided with better understanding of testing procedures. It is necessary for groups to use robust controls when testing for adventitious agents to ensure false positives are not detected.

7. Methods for the prevention of adventitious agent contamination

While this manuscript has described how adventitious agents can be detected within pharmaceutical products it is preferable to prevent the agents from contaminating the product. This can be accomplished by either removing potential contaminating agents from the product or by using manufacturing methods that are at low risk of adventitious agent contamination.

7.1. Inactivation of contaminating agents

Infectious organisms are susceptible to a variety of inactivation methods such as heat, radiation, or chemical treatment. These inactivation methods are often used on raw materials to ensure their safety, but they are also occasionally used on the products themselves. Some common examples of inactivation on raw materials include gamma irradiation of FBS and trypsin, which are performed by the manufacturers who produce the materials and have been shown to inactivate contaminating viruses [105,106]. In addition to use on raw materials, inactivation methods such as irradiation and heat treatment have been shown to be effective at inactivating adventitious agents in the production process. Treatment of products with high temperature short time treatments can allow for inactivation of adventitious agents without degrading the structure of the media [107]. Similarly, studies have shown the effectiveness of ultraviolet radiation for the inactivation of adventitious agents without the media losing its effectiveness for the culture of CHO cells [108].

Another way adventitious agents are inactivated is when a final product is delivered as inactivated, especially as formalin inactivated vaccines. A common practice in vaccine production was to culture and purify a virus, then inactivate the virus with formalin for use as a vaccine. This method of formalin inactivation also would inactivate any contaminating adventitious agents that might be present in the vaccine. Previously, when SV-40 was found to be contaminating an inactivated polio vaccine, there was evidence that the formaldehyde also inactivated the SV-40 [8].

7.2. The use of cGMP manufacturing procedures

The FDA requires pharmaceutical products to be produced using current good manufacturing practices (cGMP) to ensure safe manufacturing methods are used [109,110]. cGMPs cover a wide variety of topics including personnel, facilities, equipment, controls, and records, and also requires safe standard operating procedures be followed for each step of product production [111]. Failure to comply with cGMP procedures results in a product that is considered adulterated. cGMPs reflect the minimum standards of safe production and are broad guidelines that can be adapted by each facility as required. Generally, the use of cGMP protects products from contamination with adventitious agents during the manufacturing process and ensures proper testing protocols are in place if contamination does occur.

7.3. Avoidance of contaminated reagents

Previous experience with reagents has identified ways to avoid contamination. Animals can be screened for the presence of antibodies against common adventitious agents before they are used for material production, or the material can be screened with molecular methods prior to use. FBS can be tested for the presence of antibodies against Bovine Viral Diarrheal Virus (BVDV), the presence of which would indicate that the cattle have been exposed to BVDV [112]. Materials can also be sourced from countries or areas where certain viruses are not naturally circulating. Traditionally, FBS isolated from New Zealand and Australia has been considered to contain fewer adventurous agents than FBS from other parts of the world [113]. However, recent studies have failed to identify an advantage to geographic specific sourcing of FBS indicating that this practice is not sufficient to prevent contamination [114].

7.4. Avoidance of using animal/animal products

Since many instances of adventitious agent contamination happen through the use of contaminated animal products, avoiding the use of animal products can decrease the risk of adventitious agent exposure. This can be accomplished by making slight alterations to the manufacturing process: if a non-adherent cell line is used, porcine-derived trypsin is not required to passage the cells. It is also possible to avoid the use of FBS in culture with modifications to the media,

although the modifications required are dependent on the cell line being cultured [115]. This media is referred to as animal origin free (AOF), serum-free media (SFM), or xeno-free media. One of the main areas currently benefiting from the use of AOF or SFM is the culture of mesenchymal stem cells, with FDA approval for the use of SFM in clinical trials involving the culture of mesenchymal stem cells [116,117]. The use of serum free cell culture techniques is an emerging method that will likely see wide scale adoption as costs are reduced and the methods are improved.

Alternatively, if products can be designed to be produced in non-mammalian cell culture then the risk of advantageous agent contamination is lowered. The fermentation of yeast or bacteria is less at risk of adventitious agent contamination compared to mammalian cell culture since pathogens of yeast typically are not pathogens of humans. Insulin can be produced using the fermentation of *Saccharomyces cerevisiae* yeast by the modification of the secreted proinsulin molecule [118]. This modification allowed for the production of recombinant human insulin in fermentation culture, as opposed to isolating insulin from the pancreas from slaughtered animals. Recently, similar advances have been made in the field of monoclonal antibody production to allow for the production of monoclonal antibodies through the culture of *Pichia pastoris* yeast [119,120]. Current efforts are focused on ensuring proper glycosylation of the yeast produced antibodies to allow full functionality, but advancements could lead to monoclonal antibody therapeutic production without the need for mammalian cell culture.

8. Concluding statement

With the development of novel therapeutics, the need for efficient detection of adventitious agents is needed to ensure the safety of pharmaceutical products. No single existing method constantly detects the wide range of potential contaminating adventitious agents. Safe manufacturing processes as well as effective screening of samples is required to ensure that adventitious agent contamination is either prevented or detected before people are put at risk of adverse health events.

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