1. History of vaccines:

Vaccination is a very old, most promising and less expensive means for prevention of variety of infectious diseases caused by microorganisms [1,2]. Edward Jenner, the founder of vaccinology, first developed concept vaccination with vaccinia virus to prevent cowpox in 1796, rendering him immune to smallpox. First smallpox vaccine was developed in 1798 and later on, with systematic implementation of vaccination programs, the disease got globally eradicated in 1979. The last detection of small pox was in Somalia in 1977. In 1980, WHO declared complete eradication of small pox. In later few decades, up to 1950, many vaccines such as cholera vaccine, anthrax vaccine in humans, plague vaccine, the Bacillis-Calmette-Guerin (BCG) vaccine, tetanus, diphtheria and pertussis vaccine were developed [2].

The beginning of tissue culture for growing viruses occurred from 1950-1985 and then, led to the development of the Salk (inactivated) polio vaccine and the Sabin (live attenuated oral) polio vaccine. A systematic implementation of polio immunization programs could practically eradicate the disease globally (Figure-1). The incidences of major human diseases like smallpox, diphtheria, tetanus, yellow fever, whooping cough, polio and measles have been significantly reduced by using vaccines.

![Figure-2.1: Global scenario of Polio disease.](image-url)
2. Measles, Mumps and Rubella vaccines: [3]

Measles:

Measles is a highly contagious disease mostly affecting humans. Although majority people with measles recover after completion of natural course of infection, vaccination is the best way to prevent this disease. In 1980, measles was responsible for 2.6 million deaths per year and it is still a major disease of childhood deaths. In 2015, approximately 134 200 people, largely children below 5 years of age, died from measles in 2015 [4,5]. Systematic global implementation of Measles vaccination has reduced worldwide measles deaths between 2000 and 2015 by 79%. During 2000-2015, approx. 20.3 million lives were saved due to measles vaccination [6]. In November 2010, WHO declared the strategy of eradication of Measles with the use of vaccination.

Rubella:

Rubella (German Measles) is a comparatively milder disease. In pregnant women Rubella infection can result in birth of babies with various defects or it can also cause miscarriage/stillbirth. Rubella incidences in US are now rare due to vaccination. Rubella infection can be best controlled by vaccination. Rubella component is a part of MMR vaccine, which also protects against measles and mumps. [2]

Mumps:

Mumps disease predominantly occurs in childhood, most commonly at the age of 5 to 9 years. It is less contagious disease and there is also no specific treatment is available for mumps. First inactivated vaccine was successfully used in the 1940s and it was replaced by a live attenuated vaccine. It offers 95% protection with no adverse reactions associated with the vaccine. Mumps component is a part of MMR vaccine, which is now routinely given in many countries to all infants. As per CDC, global percentage of Measles, Mumps & Rubella infections have been significantly reduced with the use of vaccination [2]. The data of epidemiological survey made by CDC is given in Figure-2.2 to Figure-2.7 [7].
Figure-2.2: Measles cases

Figure-2.3: Measles deaths
Figure-2.4: Mumps cases

Figure-2.5: Mumps deaths
There are several global immunization / eradication vaccination programs undertaken by international agencies such as UNICEF, PAHO which obtain vaccines against the targeted diseases by various manufacturers. Measles, Rubella and Mumps vaccine have
been included as some of the major vaccines in such campaigns and millions of doses of these vaccines are expected to be required across the world for upcoming years. The manufacturing processes as well as testing methods of these viruses are well standardized. As such, it was prudent to select these vaccines for evaluation of various technologies to improve their safety with respect to unknown adventitious agents potentially entering into the product.

3. Tissue culture derived human vaccines: [9]

Before invention of tissue culture methods, viruses were being propagated by *in-vivo* method with the use of various laboratory animals such as rabbits, mice, rats and such as chicken embryonated eggs. The animal models have certain disadvantages that, they are difficult and expensive to maintain, also there is difficulty in choosing of animals for particular virus and they were not always safe and contained a whole range of nonspecific proteins. Embryonated hen’s eggs are still being used for some of the vaccines; however the disadvantages such as insufficient supply of eggs, insensitivity for many viruses, and varying choice of the site of inoculation with different viruses make this technology practically difficult as a widely accepted choice. In 1907, Ross Harrison first successfully prepared animal cell culture using frog’s embryonic nerve tissue. However, major developments in cell culture occurred in 1940s and 1950s, after which various cell cultures became scientific tools, even at large scale for manufacturing of biological products. In later period, various vaccines such as small pox, polio, rabies, measles, rubella, mumps, influenza, yellow fever were produced using tissue culture technology and successfully used in global vaccination campaigns. Today, advanced technologies such as recombinant products, fermentation of suspension cell cultures are being established for manufacturing of newer products. However, the tissue culture based vaccines still contribute a major share in the available vaccines manufactured by various technologies. Tissue cultures are relatively easy, cheaper and having broad spectrum sensitivity; however, majority of these processes use raw materials from animal origin in the manufacturing procedures. These raw materials pose a significant threat of inadvertently adding unwanted contaminants, often referred to as ‘Adventitious Agents’, to the apparently safe life-saving finished products.
4. Adventitious agents:

WHO has defined adventitious agents as microorganisms that have been unintentionally introduced into the manufacturing process of biological products. The predominant adventitious agents in tissue culture based vaccines include bacteria, fungi, yeasts, mycoplasmas, TSE agents, and viruses [10].

4.1 Sources:

The potential sources of adventitious agents in viral vaccines are cell substrates, seed virus stocks, animal derived raw materials such as foetal bovine serum, trypsin, gelatin, albumin, non-animal origin materials like amino acids, sugars, SPF eggs, etc. and contamination during handling of cells and media [10]. The cell banks have potential to vertically transmit latent viruses like herpes virus or endogenous retrovirus to next generations which can get expressed unexpectedly as an infectious virus.

4.2 Types: [11]

Bacteria and fungi/molds are present everywhere and can very well in cell culture media. Typically bacteria are 0.5–5.0 µ in size. Because of their size and fast growth rates, these microbes are most commonly encountered cell culture contaminants. The propagation of cell cultures without any antibiotics can easily detect contamination by these microbes with signs such as change in pH, turbidity and destruction of cells. These microbes can be very well detected even with a very low quantum, by carrying out sterility testing using conventional media which are used to grow these organisms and all these organisms can be identified by robust methods such as staining, morphological & biochemical characters. This makes their detection very easy and reliable. Further, these organisms can be eliminated by heat inactivation (autoclaving) and/or by applying robust filtration technologies using bacteriological (0.2 micron) filters. As such, the risk of these adventitious agents into the product can be largely eliminated by employing such detection methods.

Mycoplasmas are the Mollicutes class of bacteria. These are cell wall free organisms, typically about 0.1-0.5 microns in size and vary in form from round to
filamentous. Due to absence of cell wall, mycoplasma can squeeze to pass through bacteriological filters [12]. Majority of mycoplasma species are fastidious in nature and require nutritionally rich media for growth, although few species like *Acholeplasma* require limited nutrients and can even replicate in water [13]. Robinson & coworkers were first to detect Mycoplasma contamination in cell cultures in 1956 in HeLa cells. Mycoplasma can easily grow cell cultures attaining high densities in cell cultures. Such contaminations often occur without any signs like turbidity, change in pH or cytopathic effects. Animal derived products like sera and enzymes are major sources of Mycoplasmas, as they heat sensitive and cannot be sterilized by heat treatments like autoclaving. Conventional 0.2 micron bacteriological filters cannot completely remove mycoplasmas. However, use of a series of 0.1 micron filters can reliably eliminate the risk of mycoplasmas. Furthermore, mycoplasma contamination can be detected by carrying out testing of the process samples at various manufacturing stages using specific media and also by advanced methods such as PCR-ELISA test with a sensitivity of detecting mycoplasma to 1 cfu/ml. PDA Technical Report No.50 gives an excellent review and consideration for alternative methods for mycoplasma testing [14].

Prion proteins exist in at least two forms, the normal or cellular version and the disease causing one. The disease causing prions have ability to change normal prions into more disease causing prions, which cannot be removed by the cell’s machinery and can result in fatal form of spongiform encephalopathy. This transmissible spongiform encephalopathy has been reported in deer, elk, cattle, sheep and goats. TSE has also been found in cats that ingested infected bovine material in a zoo setting (Feline SE). TSE in humans is found as an inherited disease (Fatal Familial Insomnia), as a spontaneous occurrence (Creutzfeldt-Jakob Disease (CJD)) or as an infectious disease via ingested infected bovine meat (vCJD). A second route of acquiring vCJD is through a blood transfusion from a vCJD infected donor [12].

Bovine spongiform encephalopathy (BSE) agents are associated with bovine sera, which are commonly used as a growth supplement for tissue cultures. BSE is a prion disease involving neurological disorders in adult cattle. These are difficult to detect by reliable testing methods. Since the process that inactivates prions is extreme (EMEA/410/01 Rev 2), the sourcing is a more practical approach for control. World Organization for Animal Health (OIE) conducts frequent surveillance of BSE cases
across the globe and declares a list of countries with a BSE risk category (www.oie.int/animal-health-in-the-world/bse-portal/). The risk of these adventitious agents can be minimized by proper sourcing of such raw materials from selected geographical locations (e.g. Australia, New Zealand) by referring the OIE list [12].

Amongst all the types, the viruses are considered to be most risky adventitious agents. Viruses are obligate parasites at the genetic level, thus their replication is strictly dependent on the host’s biochemical machinery. Viruses unlike other adventitious agents must have a cell to replicate in. Viruses are structurally simple and are made up of a genome that can be either RNA or DNA, a few proteins and some have a lipid envelope acquired from the host. Viruses cannot be viewed by light microscopy and range in size from 200nm to 17nm [15]. Their common sources are cell banks, specific pathogen free eggs, seed viruses and other animal derived raw materials.

4.3 Examples of product contaminations: [15]

The risk of introduction of viral adventitious agents in tissue culture based vaccines has been a major problem. For example, yellow fever vaccine was detected with hepatitis B virus introduced through human-derived excipient, polio and adeno virus vaccines were found to be contaminated with SV-40 virus arising from primary monkey kidney cells. Endogenous avian retrovirus in some egg derived vaccines, porcine circovirus in live attenuated Rotavirus vaccine due to porcine trypsin (Rotarix of GSK and Rotateq of Merck) are some more examples of adventitious agents in vaccines. At least ten virus families, originated from raw materials, were detected in CHO cells, which are used as a substrate for many biological products.

To date, there are no cases of human disease after use of vaccines which are known to be attributable to such adventitious agents. However, such contaminants need to be considered as a potential risk and the safety of these products can very well be achieved with the help of extensive virus testing and validation of virus removal or inactivation methods.
4.4 Control:

The control of adventitious agents starts with the raw material vendor and ends with the biopharmaceutical user. Some common raw materials include Bovine serum, recombinant proteins (e.g. insulin), peptones of various sources, salts, amino acids and sugars stores in large quantities (with a potential of contamination by rodents, insects during shipping & storage). There are three principal approaches to control the potential viral contamination of biotechnology products [16]:

- Appropriate choice and testing of starting materials (cell banks and consumables, etc.)
- Testing at intermediate steps of manufacturing process
- Establish a validated virus inactivation and or removal steps into the production processes.

Taking into consideration the past experience over the years, various international guidelines are getting more stringent from time to time regarding extensive characterization of cell banks and virus stocks prior to their use for production of vaccines. Such testing can minimize the risk of adventitious agents from cell banks and virus stocks. In case of cell banks, characterization of cell banks after growing them up to production stage further minimizes such risk. Testing of specific pathogen free eggs for all potential adventitious agents and monitoring of flocks for a series of generations ensures their safety with respect to adventitious agents including vertically transmitted viruses. Animal derived raw materials, being consumables, are having lot to lot variation with respect to the risk of adventitious viruses and are therefore considered to be major potential source of such contaminants.

5. Global regulations for raw materials:

Several methodologies have been suggested to detect & possibly eliminate the risk of such contaminants being present in the raw materials. Sourcing of the raw materials is an important part of regulatory documents. Appropriate sourcing can minimize risk of prions. Other potential contaminants such as mycoplasma, bacteria and molds/yeasts can be taken care by various treatments like heat, pH changes, filtration,
irradiations, etc.; however such methods need to be established by validation. A science based approach based on the characteristics of the adventitious agents is required to control contamination by viruses, bacteria, mycoplasma and molds/yeasts, prions. Global regulations, current publications and current PDA technical reports aid in the development of this approach [11]. Global regulations that give guidance on virus detection that can be applied to raw materials include the USDA 9 CFR 113.53 and 113.47, ICH Q5A, EMA/CHMP/BWP/398498/2005, CBER Guidance for Industry - Cell Substrates and Biomaterials and the Japanese Pharmacopeia 210 [16,17,18,19,20,21].

6. Trypsin & FBS:

Trypsin and FBS are the two most essential animal derived raw materials for manufacturing of majority of the tissue culture based vaccines. Recent European regulatory agencies provide stringent guidelines for trypsin & FBS for minimizing risk of adventitious agents.

Conventionally, porcine trypsin extracted from the pancreas of pigs is used. (Marcus-Sekura et al., 2011). There are many porcine viruses potentially infectious to human species, which can grow in Vero cells and/or porcine cells. The guidelines suggest use of two different cell lines to cover up such viruses. Besides testing of trypsin, it is also suggested to have virus inactivation/removal steps in the manufacturing process. It is also recommended to explore possibilities of using animal component free reagents in cell cultures such as recombinant, bacterial or plant-derived trypsin, which can completely eliminate the risk of adventitious agents [22].

Foetal bovine serum is originated from healthy cattle certified to be suitable for human consumption. FBS is considered as the most common potential source of adventitious agents. The Office International des Epizooties (OIE) code, which defines the status of country, needs to be followed for sourcing of FBS. The testing for adventitious viruses need to be done after 0.1 micron filtration, but before any steps of inactivation or removal of viruses. Current regulatory guidelines recommend testing of FBS as per 9CFR testing procedures, which includes testing of most probable contaminants of FBS. BVDV is a very ubiquitous infection of cattle and the sera almost
invariably show some antibody level indicative of past infection. In recent years, EMA has also considered bovine polyoma virus as one of the new contaminants. However, due to lack of reliable testing methods, EMA does not make it mandatory to test, but it warns serum manufacturers to be alert of this concern. In order to minimize risks associated with FBS, it is strongly recommended to introduce validated methods of virus inactivation and / or removal use in the process. Gamma irradiation is one of the recommended method this purpose [23,24].

7. References:

7 CDC. “Reported Cases and Deaths from Vaccine Preventable Diseases, United States, 1950-2013” cdc.gov, Sep. 2014.


Guideline on the use of bovine serum used in the manufacture of human biological medicinal products; May 30, 2013, EMA/CHMP/BWP/457920/2011; Committee for Medicinal Products for Human use (CHMP).