



Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.



Review

Extraneous agent detection in vaccines – A review of technical aspects

Attila Farsang*, Gábor Kulcsár

National Food Chain Safety Office, Directorate of Veterinary Medicinal Products, Budapest, Hungary

ARTICLE INFO

Article history:

Received 11 September 2011

Received in revised form

26 March 2012

Accepted 17 April 2012

Keywords:

Vaccine

Contamination

Viral safety

Novel molecular methods

ABSTRACT

The quality and safety of commercial vaccines have a profound importance. Contrary to all precautions and efforts the use of biological material in vaccine development and production may lead to potential contamination of the vaccines with known and unknown extraneous agents (EAs).

In veterinary field official lists of EAs have been compiled as legal framework to describe the potential agents, which must be tested during manufacture of vaccines. Nevertheless, detection of known and unknown contaminants in vaccines is a common duty for manufacturers and authorities of both veterinary and human field sharing similar needs of special technical approaches. State-of-art molecular methods such as randomly primed PCR combined with massive parallel sequencing (MPS) or microarrays may open new perspectives in extraneous agent testing. The robustness and efficacy of this technical approach in vaccine control was clearly demonstrated on a human vaccine example when porcine circovirus type 1 (PCV1) contamination was revealed in Rotarix, a human rotavirus vaccine. The consequences and implications are reviewed hereby from a veterinary regulatory point of view.

© 2012 The International Alliance for Biological Standardization. Published by Elsevier Ltd. All rights reserved.

1. Introduction

The benefits of vaccination are unquestionable as vaccines are in general one of the most effective means of preventing infectious diseases in both animals and humans [1]. In 2010 the 30th anniversary was celebrated that smallpox had been eradicated, while recently the global freedom from Rinderpest was announced by the Food and Agriculture Organisation of the United Nations (FAO) underlining the remarkable achievements of vaccines and vaccination. In veterinary science vaccines help to protect health and welfare of the animals and prevent spread of diseases to humans.

Animal diseases are responsible for at least 17% loss in production and reduction of these losses has a paramount importance [2]. Considering the fact that the European Union applies pre-emptive culling to handle the outbreaks of highly contagious so-called transboundary animal diseases (TADs) the costs of TAD outbreaks are tremendous, because of animal health measurements applied, destruction of carcasses and compensations paid to farmers. The estimated costs of the UK foot-and-mouth disease (FMD) outbreaks in 2001 were approx. £3.800 million (USD 5.186 million) [3,4], while avian influenza (AI) outbreaks in the Netherlands in 2003 cost at least USD 612 million. Classical swine fever (CSF) outbreaks

in the Netherlands between 1997 and 1998 caused USD 2.52 billion losses. Whenever it is possible the “vaccination to live” policy should be followed, because mass slaughter of animals in an outbreak has not only extremely high costs, but it is also rejected by the society on ethical basis and [2].

Compared to the high costs of TAD outbreaks the development of a brand new vaccine needs approx. USD 50 million, nevertheless the whole process may take eight to ten years including demonstration of the safety and efficacy of the new product [2].

The viral safety of vaccines is a key issue for both veterinary and human vaccines, which implies assurance of freedom of medicinal products from agents other than stated on the label. Furthermore, viral safety regarding vaccines also means that residual pathogenicity is within acceptable limits for safe use and the inactivated agents are completely inactivated.

Viral safety may be jeopardised by the following main factors: i.) reversion to virulence; ii.) recombination; iii.) incomplete inactivation; iv.) contaminations with EAs [5]. Vaccine development and production are profoundly linked to the use of biological material of animal or human origin, such as cell cultures obtained from various sources and foetal calf serum (FCS) used for growing the cells. The use of such biological material in vaccine development and production may lead to potential contaminations of the vaccine products. The contamination may happen in various steps of the manufacture procedure including the starting materials (master and working seed viruses), the production procedure itself and forming of finished product.

* Corresponding author. Tel.: +36 1 433 0330; fax: +36 1 262 2839.
E-mail address: farsanga@oai.hu (A. Farsang).

To ensure viral safety of animal and human medicinal products, regulations and guidelines have been developed [6], moreover official lists of EAs have been compiled by Ph. Eur (see chapters 2.6.24 and 2.6.25) [7] and European Medicines Agency (EMA) to describe the potential agents, which must be tested during manufacture of vaccines.

The methodology how to test vaccines and how to detect EAs has similarities between the veterinary and human fields. The recent case and its technical background when PCV1 was revealed in a human vaccine offer several general implications for vaccine control and EA detection, which are discussed and reviewed hereby from a veterinary authority point of view.

2. Categories of EAs

The number of identified viruses reaches approximately 2300 viral species. However, the likely number of viruses on our globe may exceed 150,000 [8]. Based on another calculation this number could be even more striking, if you presume that every vertebrate animal has some 20 endemic viruses, then, given the fact that there are over 50,000 vertebrate species. Obviously, all of them cannot be found, but it is highly advisable to screen at least those animals that we come into contact with most and to obtain more knowledge about what viruses they may harbour [9].

When discussing viral diversity and evolution, it is also very important to note that viruses evolve on a regular basis, constantly fuelled by mutations, which lead to complex mutant distributions, termed viral quasispecies. Quasispecies can ensure high genetic flexibility allowing rapid adaptation to environment [10]. Accordingly, the vast majority of viruses still belong to the “unknown” emerging virus category today.

Considering the viral EAs, three categories can be distinguished: i.) “known known” agents that are both known and suspected in the sample and should be tested for; ii.) the “known unknown” agents, which are known and can be tested for, but are not necessarily suspected in the sample; and iii.) the “unknown unknown” agents, which are yet unknown and cannot be currently tested for [5].

3. Vaccine contaminations

Contrary to all unequivocal advantages of vaccines and vaccination, both the past and the present offer several examples when a certain vaccine was contaminated [8]. Foamy virus (Spumaretroviridae) was identified as a contaminant of primary monkey kidney cultures used for vaccine production in the early 1950s. The virus causes a foam-like syncytial effect in cell cultures leading to cell lysis and destruction of cell cultures. However, they do not cause any clinical symptoms *in vivo*. Infected animals induce life-long persistent infection [11]. In the 1960s it was shown that yellow fever live attenuated vaccines prepared in chicken embryo fibroblasts were infected with avian leukosis virus (ALV) [12]. Nevertheless, no increased risk for cancer was observed in vaccinated individuals, which was expected considering that ALV does not grow efficiently in mammalian cells [13–15]. Calicivirus was found in Chinese hamster ovary (CHO) cells [16]. Jorgensen et al. [17] revealed Newcastle disease virus (NDV) vaccine strains in different live poultry vaccines, while in 1990 a live attenuated multi-component canine vaccine was contaminated with a serotype of bluetongue virus causing abortions and death in pregnant bitches [18,19]. Pestiviruses (BVDV types 1 and 2) are one of the most common EAs in veterinary and human vaccines [20–22,24,25]. The potential source of pestivirus in live vaccines prepared for human and veterinary use may be the use of FCS, which is applied for growing the cells during vaccine production

[23]. As a further threat besides BVDV type 1 and 2 *novel bovine pestiviruses* (BVDV 3) were also detected in foetal calf serum used by diagnostic laboratories and bioindustry. These batches usually originated from Brazil, while contaminated bovine serum samples came from Thailand [26–29]. The diagnostic problem posed by novel bovine pestiviruses is primer sets designed to BVDV 1 and 2 usually fail to detect BVDV 3 sequences.

Another recent EA is RD114, a replication-competent feline endogenous γ -retrovirus with xenotropic host spectra [30], which was found in feline parvovirus vaccine [31], canine distemper, adenovirus type 2 and parvovirus type 2 vaccines [32]. RD114 can be originated from the use of endogenous retrovirus susceptible cell lines, for instance Crandell-Rees feline kidney (CrFK) cells [33], which are widely applied to produce dog vaccines, especially against canine coronavirus and parvovirus.

The most notable case of human vaccine contamination to date may have been when in the 20th century tens of millions of people worldwide were immunised with polio vaccines containing simian virus 40 (SV40) [34,35]. SV40 was found to cause cancer in animals and is associated with human brain, bone and lung cancers, however, a clear connection was not found between this certain vaccine and any human tumour cases [36].

The debate of what has to be considered as EAs and how to handle them from a regulatory point of view has been re-burdened very recently, when a porcine circovirus 1 (PCV1) was found in a rotavirus vaccine widely used worldwide for children [37]. This case triggered not only the paramount interest of the scientific and regulatory community, but also positioned vaccines and vaccine biosafety to immense public attention (<http://edition.cnn.com/2010/HEALTH/03/22/rotavirus.vaccine/index.html>).

Rotarix is a genetically engineered vaccine to prevent diarrhoea caused by rotavirus infection. It was created by GlaxoSmithKline (GSK) by isolating human rotavirus strain infecting a child in Cincinnati and using African Green monkey kidney cells to produce the original viral seed stock from which all Rotarix vaccine has been made [38]. It was licensed by the US Health Authority, the Food and Drug Administration (FDA), in 2008 and besides US it is used in altogether 114 countries including EU member states. Approximately 30 million children worldwide (primarily in Latin America) and a further approx. 1 million in the United States were vaccinated with Rotarix.

The EA was PCV1 – A Circovirus first described by Tischer et al. [39] as a contaminant of porcine kidney cell culture – which was first revealed in Rotarix by an academic research team using metagenomics and microarray tests [37]. Their aim was to evaluate the purity of eight live virus vaccines for polio, rubella, measles, yellow fever, human herpes (varicella or chicken pox), rotavirus (Rotarix and Rotateq) and multivalent measles-mumps-rubella (MMR) vaccine [40].

In the FDA licensing process, Rotarix had met FDA standards, which included demonstrating the freedom from Transmissible Spongiform Encephalopathy (TSE) and certain bovine viruses, because bovine serum was used to prepare the original viral seed stock [41]. Porcine trypsin was also used to produce the viral seed stock [42]. GSK confirmed the presence of PCV1 in both the cell bank and the seed from which the vaccine is derived, suggesting its presence from the early stages of vaccine development.

In concordance with the fact PCV1 is considered harmless to humans even it does not cause disease in its natural host, in swine, no unexpected pattern of serious adverse reactions to Rotarix has been reported in any country. Nevertheless, subsequently FDA learned about the contamination, doctors were advised not to use this vaccine [43]. At the same time public health officials emphasised the suspension of Rotarix usage applied only to the United States and a risk-benefit approach should be followed in

countries where the incidence of rotavirus is more severe. Rotavirus disease kills more than 500,000 infants around the world each year, mainly in low- and middle-income countries and before a rotavirus vaccine became available the disease was blamed for more than 50,000 hospitalizations and several dozen deaths per year in the United States [43]. Thus, the usage of the contaminated Rotarix may be justified when the benefits of vaccination outweigh any concerns due to the contamination.

Besides Rotarix vaccine, another vaccine against rotavirus was found contaminated [37]. DNA fragments of simian retrovirus (SRV) were revealed in Rotateq vaccine, which is a genetically engineered product of Merck. Rotateq contains five human-bovine reassortment strains of rotavirus that were created at the Children's Hospital of Pennsylvania (CHOP), where rotavirus strains causing bovine diarrhoea were combined with human strains of rotavirus. The reassortment viruses were transported to Merck, where master seeds were produced using African Green Monkey kidney cell cultures. Foetal bovine serum and porcine trypsin were also added to produce the seed stock [44]. Small amounts of residual bovine serum and cell culture media were also revealed in Rotateq vaccine [44,45].

The significance of "silent" presence of potential contaminants in vaccines cannot be fully estimated based on the available data at present. However, the artificial introduction of certain agents into a new host may cause a very complicated virological situation. It is clearly underlined by the examples, when vaccines for pigs contaminated with circovirus may be played a role in onset and spread of postweaning multisystemic wasting syndrome (PMWS) or the inactivated vaccines produced from sheep brain to protect sheep against louping ill, a brain inflammatory ailment spread by ticks, had a clear relation in the escalated transmission of scrapie [46].

4. Improvements in methodology of viral detection

Cell culture is a central way of viral detection, which facilitates downstream analysis with other laboratory methods including electronmicroscopy (EM), serology and PCR. Detection of antibodies induced by a certain agent in a host is also a conventional approach [47].

The conventional detection methods are seriously hampered by the fact that many potential EAs do not replicate in cell culture, or a suitable animal model has not been identified. However, the discovery of unknown non-cultivable viruses is ongoing and several methods were used for the identification of unknown non-cultivable agents such as EM [48,49], EM combined with serology [50,51] or immune serum to screen peptide expression libraries generated from samples infected with unknown viruses [52].

Nucleic acid based detection methods are so sensitive that they detect small numbers of viruses that may occur in the absence of disease. The limitation of these methods is the need of a priori knowledge of the viral nucleic acid sequence to be detected [53]. Moreover, the interpretation of PCR positive results is also complex and often required further testing, considering the fact molecular techniques like PCR detects nucleic acids and not infectivity.

RNase/DNase digestion prior to PCR both improves the sensitivity of detection by removal of background DNA and as the nucleic acid in the virion is protected from DNase/RNase, the obtained PCR positivity profoundly indicates the presence of intact (perhaps infective) virions [54]. This may help to address the question if only non-infective genomic fragment or a native virion were detected by PCR.

Besides traditional PCR many variants of the real-time PCR assays had been developed. These methods highly vary in which chemistries are used e.g. TaqMan, molecular beacons (MB),

scorpion primers, dual probe systems, dye-labelled oligonucleotide ligation or Primer-Probe Energy Transfer System (PriProET) [55]. Compared to traditional PCR the real-time assays are faster, nearly equally sensitive and allow quantification. Their throughput is higher, the risk of contamination is lower as there is no need to open the test tube for post-PCR handling of the product. Real-time PCR allows automation and multiplexing, because probes can be labelled with a number of different fluorophores leading to lower diagnostic costs as final consequence [55].

In veterinary field real-time PCR are taking over traditional PCRs. PriProET was suggested having the most robust diagnostic potential among others real-time PCR variants, because PriProET needs shorter conserved region to hybridise and less fragile to single point mutation [56,57].

So-called proximity ligation offers a novel mean to detect antibody by nucleic acid amplification. In this method antibodies recognising viral or bacterial surface proteins are bound to DNA strands. These DNA strands can be ligated and subsequently amplified by a real-time PCR when antibodies are bound to surface antigens of the infectious agent [58].

Considering all different types of PCR the common feature is that the interpretation of PCR results can be difficult from a risk assessment point of view. For instance, lower risk may be posed by a contamination, which is non-specific to the species targeted by the contaminated vaccine [59]. However, the possibility of cross-species contamination may undermine the simplicity of this approach as in case of bovine spongiform encephalitis which was shown to be able to cross the species barrier between bovines and humans [59,60].

The use of most recent technical developments including sequence-independent molecular techniques combined with high-throughput sequencing or microarray analysis may improve and accelerate the viral discovery. PCV1 in Rotarix and SRV in Rotateq were revealed both by Massive Parallel Sequencing (MPS) combined with the sequence-independent nucleic acid techniques and microarray tests on RNase/DNase digested samples [37].

Both random PCR combined with MPS and microarray tests have the advantage to detect a much wider range of potential extraneous agents than the currently mandated methods like cell cultures or agent-specific PCR [37]. The strength of metagenomics is that this method does not target any specific virus/pathogen, but has a general capacity to detect a wide range of infectious agents, including unknown emerging novel viruses. The advantage of microarrays is the potential of highly multiplexed detection of EAs. Numerous microbes can be simultaneously detected by microarray using ≥ 70 nucleotides long probes provided melting temperatures are high enough to ensure hybridisation despite the restricted complementary between probe and target.

Nevertheless, both metagenomics and microarray test have their own limitations. PCR is more sensitive than either metagenomics or microarrays when screening for specific viruses. Without the pre-amplification of the clinical sample most microarrays are not sensitive enough [61]. The use of random PCR step prior to microarray hybridisation can improve the sensitivity of microarrays enabling in the same time the detection of EAs without any a priori knowledge of the infectious agent. Considering the fact PCR-based random amplification of whole genome was demonstrated to generate incomplete coverage of genome, Erlandsson et al. [62] used isothermal multiple displacement amplification of whole genomes by the Phi29 polymerase. Phi29 polymerase allows very good coverage of the genomes because of its high processivity and extremely low error rate [62]. Phi29 polymerase can also be used for improvement of sensitivity of an agent-specific real-time PCR [63]. In the so-called AMP-PCR, an unbiased random Phi29 pre-amplification is carried out before a specific real-time PCR reaction.

Thus, AMP-PCR increases the specific PCR signal allowing the detection of positive samples normally under the detection limit of the specific real-time PCR. As AMP-PCR reaction takes place in one vessel, the risk of contamination is eliminated and nested PCR can be replaced by AMP-PCR in situations where increased sensitivity is needed e.g. in routine PCR diagnostic analysis [63].

Altogether the use of metagenomics and microarrays may offer new perspectives in the vaccine quality control allowing detection further – probably at present unknown – agents in vaccines without any a priori (genetic) knowledge of these agents, or any a priori suspicion of contamination.

5. Legislative issues

To ensure viral safety of animal and human medicinal products, regulations and guidelines have been developed [6]. To describe the potential agents, which must be tested during manufacture of vaccines, an official list of EAs has been compiled by the Ph. Eur. (see chapter 2.6.25) [7], and a relevant guideline was issued by the Committee for Medicinal Products for Veterinary Use (CVMP) [64]. The advent of molecular biology and the increased globalisation of veterinary vaccine production altogether with the increased need for a review of the progress and requirement in viral safety have led to changes in the regulatory approach.

The present guidelines and specific recommendations on extraneous agent testing of veterinary medicinal products issued by the above mentioned bodies [7,64] require that all starting materials and final products produced *in vivo* and *in vitro* must be tested for the presence of potential contaminants including viruses, bacteria, fungi and mycoplasma in addition to stability and identity where appropriate. EAs lists would need regular revision based on recent epizootological data (on both EU and US sides and including specific avian purity testing as described in Ph. Eur.) and specification of subtypes/substrains, if possible after a thorough risk assessment together with the definition of levels of contamination.

Despite of all efforts of manufacturers and competent authorities, the risk of contamination cannot be completely excluded, only the probability can be minimised, which poses the question: what should be done to increase the maximum safety? Regular revision of testing of Working Seed Viruses (WSVs) and batches/finished products should be necessary at GMP and GLP level. Information should be collected about the geographic location of the facility, quality of facilities/laboratories, quality and health status of personnel and consolidated data from epidemiological monitoring concerning the status of epizootic/enzootic diseases in the geographic origin of the starting materials. All this can lead to a reduction in the number of EAs, which should be tested within the production process. Furthermore it would be essential to define the steps of the manufacturing process where molecular methods can be applied. The reduction in number of EAs and the increasing use of molecular methods instead of traditional animal testing are in profound concordance with '3R's rule as reduce, refine and replace animal testing in the manufacture and testing of veterinary medicinal products, which also recognised by Ph. Eur. (see chapter 1.1) [7]. The Ph. Eur. already allows for the use of nucleic acid techniques provided these techniques offer comparable sensitivity and specificity to the conventional methods (see chapter 2.6.21). The new methods may have to be assessed not in comparison with the conventional and compendial methods, but rather in the light of the current needs. To reach this latter aim validation of testing methods in collaborative studies with the involvement of Official Medicines Control Laboratories (OMCL) would also be necessary. OMCLs are key players for the control of immunological and pharmaceutical products. Their significance is based on their neutral status as OMCLs belong to national competent authorities.

To avoid the contamination of cell cultures with retroviruses it would be advisable to include product-enhanced reverse transcriptase assay (PERT) in the Ph. Eur. for cell bank testing with the explicit indication that a positive test result should be further investigated [59]. The significance of PERT was demonstrated when low level reverse transcriptase (RT) activity was found in attenuated vaccines with chicken-cell origin. Subsequent investigations revealed that the detected RT activity was associated the presence of the incomplete endogenous avian retrovirus (EAV-0 and avian leukosis virus). However, these viruses proved to be non-infectious as the viral envelope protein required for viral infectivity is missing, their presence in vaccines triggered concerns regarding the safety of these vaccines [65]. These events pointed out that PERT similarly to PCR offers extreme increase in sensitivity compared to traditional methods [66]. The biological significance of the findings raises questions, because RT activity may be conceivably associated with several sources including cellular enzymes like DNA or RNA polymerases, telomerases and cellular elements like retrotransposons [65].

Revision of and development in inactivation methods for starting materials of animal origin and establishment of well controlled test systems for insect cell lines and insect viruses also have a profound significance [59].

6. Concluding remarks

The significance of a "silent" presence of potential contaminants in vaccines cannot be fully estimated. The artificial introduction of agents into a new host may cause a very complicated virological situation, for instance vaccines for pigs contaminated with PCV2 may have contributed to the occurrence of the postweaning multisystemic wasting syndrome (PMWS) or the inactivated vaccines produced from sheep brain to protect sheep against louping ill – a brain inflammatory ailment spread by ticks – clearly played a significant role in the escalated transmission of scrapie [46]. To minimise such risk and ensure high quality and safe vaccines have a profound importance. State-of-art molecular methods like metagenomics and microarrays can improve the capacity of both manufacturers and competent authorities to reveal contaminations and to identify the well-known and the potentially new EAs. The implementation of these methods into routine tests of vaccines and their acceptance by Ph. Eur. or EMA as official methods need validation. A further requirement of the acceptance is to improve interpretation of results obtained by these tests especially in comparison with those obtained by currently mandated tests. A past example underlines that the improved ability to screen vaccines for contamination has a direct relation to the improvement of vaccine quality and safety. Subsequently SV40 had been revealed in vaccines, manufacturers were requested by control authorities to test for the presence of SV40, which led to the full clearance of SV40 from vaccine batches released after 1961.

Contaminations may compromise the advantageous effects of both veterinary and human vaccines and may undermine the trust in vaccines and vaccination. The maintenance of this trust is a profound responsibility of both manufacturers and the competent authorities.

References

- [1] Andre FE. Vaccinology: past achievements, present roadblocks and future promises. *Vaccine* 2003;21:593–5.
- [2] O'Brien D, Zanker S. Animal vaccination and the veterinary pharmaceutical industry. *Rev Sci Tech* 2007;26:471–7.
- [3] Anon. Cost benefit analysis of foot and mouth disease controls. A report for the Department for Environment. United Kingdom: Food and Rural Affairs.

- Risk Solutions. Available at: <http://archive.defra.gov.uk/foodfarm/farmanimal/diseases/atoz/fmd/documents/costben.pdf>; 2005.
- [4] Blake A, Sinclair MT, Sugiyarto G. Quantifying the impact of Foot and Mouth Disease on tourism and the UK economy. *Tourism Econ* 2003;9:449–65.
 - [5] Mackay D, Kriz N. Current challenges in viral safety and extraneous agent testing. *Biologicals* 2010;38:335–7.
 - [6] Thornton DH. Quality control of vaccines. In: Alexander DJ, editor. *Newcastle disease*. Boston: Kluwer; 1988. p. 347–65.
 - [7] *European Pharmacopoeia*. 7th ed. Strasbourg: Council of Europe; 2009.
 - [8] Pastoret PP. Human and animal vaccine contaminations. *Biologicals* 2010;38:332–4.
 - [9] Lipkin WI. Microbe hunting. *Microbiol Mol Biol Rev* 2010;74:363–77.
 - [10] Domingo E. Mechanisms of viral emergence. *Vet Res* 2010;41:38.
 - [11] Meiering CD, Linial ML. Historical perspective of foamy virus epidemiology and infection. *Clin Microbiol Rev* 2001;14:165–76.
 - [12] Dougherty RM, Harris RJ. Contaminant viruses in two live virus vaccines produced in chicken cells. *J Hyg* 1966;64:1–7.
 - [13] Harris RJ, Dougherty RM, Biggs PM, Payne LN, Goffe AP, Churchill AE, et al. Contaminant viruses in two live virus vaccines produced in chick cells. *J Hyg* 1966;64:1–7.
 - [14] Urisman A, Molinaro RJ, Fischer N, Plummer SJ, Casey G, Klein EA, et al. Identification of a novel Gammaretrovirus in Prostate Tumors of patients Homozygous for R462Q RNASEL Variant. *PLoS Pathog* 2006;2:e25.
 - [15] Weiss RA. Adventitious viral genomes in vaccines but not in vaccinees. *Emerg Infect Dis* 2001;7:153–4.
 - [16] Oehmig A, Büttner M, Weiland F, Werz W, Bergeman K, Pfaff E. Identification of a calicivirus isolate of unknown origin. *J Gen Virol* 2003;84:2837–45.
 - [17] Jørgensen PH, Handberg KJ, Ahrens P, Manvell RJ, Frost KM, Alexander DJ. Similarity of avian paramyxovirus serotype 1 isolates of low virulence for chickens obtained from contaminated poultry vaccines and from poultry flocks. *Vet Rec* 2000;146:665–8.
 - [18] Levings RL, Wilbur LA, Evermann JF, Stoll IR, Starling DE, Spillers CA, et al. Abortion and death in pregnant bitches associated with a canine vaccine contaminated with bluetongue virus. *Dev Biol Stand* 1996;88:219–20.
 - [19] O'Toole D, Van Campen H, Woodlard L. Bluetongue virus: contamination of vaccine. *J Am Vet Med Assoc* 1994;205:407–8.
 - [20] Vilcek S, Herring AJ, Herring JA, Nettleton PF, Lowings JP, Paton DJ. Pestivirus isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. *Arch Virol* 1994;136:309–23.
 - [21] Giangaspero M, Vacirca G, Harasawa R, Buttner M, Panuccio A, de Giuli Morghen C, et al. Genotypes of Pestivirus RNA detected in live virus vaccines for human use. *J Vet Med Sci* 2001;63:723–33.
 - [22] Kulcsar G, Farsang A, Soos T. Testing for viral contaminants of veterinary vaccines in Hungary. *Biologicals* 2010;38:346–9.
 - [23] Erickson GA, Bolin SR, Landgraf JG. Viral contamination of fetal bovine serum used for tissue culture: risks and concerns. *Dev Biol Stand* 1991;75:173–5.
 - [24] Falcone E, Tollis M, Conti G. Bovine viral diarrhoea disease associated with a contaminated vaccine. *Vaccine* 2000;18:387–8.
 - [25] Levings RL, Wessman SJ. Bovine viral diarrhoea virus contamination of nutrient serum, cell cultures and viral vaccines. *Dev Biol Stand* 1991;75:177–81.
 - [26] Liu L, Xia H, Belák S, Baule C. A TaqMan real-time RT-PCR assay for selective detection of recently described bovine atypical pestiviruses in clinical samples and biological products. *J Virol Meth* 2008;154:82–5.
 - [27] Liu L, Kampa J, Belák S, Baule C. Virus recovery and full-length genome analysis of the atypical bovine pestivirus Th/04_KhonKaen. *Vet Microbiol* 2009;138:62–8.
 - [28] Schirmer H, Strebelow G, Depner K, Hoffmann B, Beer M. Genetic and antigenic characterization of an atypical pestivirus isolate, a putative member of a novel pestivirus species. *J Gen Virol* 2004;85:3647–52.
 - [29] Ståhl K, Kampa J, Alenius S, Persson Wadman A, Baule C, Aiumlamai S, et al. Natural infection of cattle with an atypical 'HoBi'-like pestivirus -Implications for BVD control and for the safety of biological products. *Vet Res* 2007;38:517–23.
 - [30] Reeves RH, O'Brien SJ. Molecular genetic characterisation of the RD-114 gene family of endogenous feline retroviral sequences. *J Virol* 1984;52:164–71.
 - [31] Miyazawa T, Yoshikawa R, Golder M, Okada M, Stewart H, Palmarini M. Isolation of an infectious endogenous retrovirus in a proportion of live attenuated vaccines for pets. *J Virol* 2010;84:3690–4.
 - [32] Yoshikawa R, Sato E, Miyazawa T. Contamination of infectious RD-114 virus in vaccines produced using non-feline cell lines. *Biologicals* 2011;39:33–7.
 - [33] Baumann JG, Gunzburg WH, Salmons B. CrFK feline kidney cells produce an RD114-like endogenous virus that can package murine leukaemia virus-based vectors. *J Virol* 1998;72:7685–7.
 - [34] Hilleman MR. Discovery of simian virus 40 (SV40) and its relationship to poliomyelitis vaccines. *Dev Biol Stand* 1998;94:183–90.
 - [35] Shah K, Nathanson N. Human exposure to SV40: review and comment. *Am J Epidemiol* 1976;103:1–12.
 - [36] Stratton K, Almario DA, McCormick MC, editors. *Immunisation safety review: SV40 contamination of polio vaccine and cancer*. National Academies Press; 2002. p. 102.
 - [37] Victoria JG, Wang C, Jones MS, Jaing C, McLoughlin K, Gardner S, et al. Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. *J Virol* 2010;84:6033–40.
 - [38] FDA. Memorandum: review of Vero cell banks used for vaccine production and adventitious agent testing of virus seeds and vaccine human rotavirus vaccine (HRV) – Rotarix. <http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm134138.htm>; April 1, 2008.
 - [39] Tischer I, Rasch R, Tochtermann G. Characterisation of papovavirus- and picornavirus-like particles in permanent pig kidney cell lines. *Zbl Bakt Hyg Abt Orig A* 1974;226:153–67.
 - [40] Racaniello V. Deep sequencing reveals viral vaccine contaminants, *www.virology.ws*; March 29, 2010, http://www.virology.ws/2010/03/29/deep-sequencing-reveals-viral-vaccine-contaminants/?utm_source=feedburner&utm_medium=email&utm_campaign=feed%253a+virologyblog+%2528virology+blog%2529%0a; March 29, 2010.
 - [41] FDA. Summary basis for FDA regulatory Action – Rotarix. <http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm133543.htm>; June 4, 2007.
 - [42] European Medicines Agency. Evaluation of Medicines for human use: Rotarix vaccine (Control of materials (Reagents) page 4). http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000639/WC500054789.pdf; 2006.
 - [43] FDA. News release: components of extraneous virus detected in Rotarix vaccine: no known safety risk, FDA recommends clinicians temporarily suspend use of vaccine as agency learns more. <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm205625.htm>; March 22, 2010.
 - [44] European Medicines Agency. Evaluation of Medicines for human use: Rotateq vaccine (Active Substance (Manufacture)). Pages 3–10. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Summary_for_the_public/human/000669/WC500054181.pdf; 2006b.
 - [45] FDA. Memorandum: clinical review of New Biologics License application – Rotateq (Description of the product). Page 12. <http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM142304.pdf>; April 6, 2005.
 - [46] Pastoret PP, Blancou J, Vannier P, Verschuere C, editors. *Veterinary vaccinology*. Elsevier; 1997.
 - [47] Tang P, Chiu C. Metagenomics for the discovery of novel human viruses. *Future Microbiol* 2010;5:177–89.
 - [48] Caul EO, Appleton H. The electron microscopical and physical characteristics of small round human fecal viruses: an interim scheme for classification. *J Med Virol* 1982;9(4):257–65.
 - [49] Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J Virol* 1972;10:1075–81.
 - [50] Blumberg BS, Alter HJ, Visnich S. A 'new' antigen in leukemia sera. *JAMA* 1965;191:541–6.
 - [51] Dane DS, Cameron CH, Briggs M. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet* 1970;1:695–8.
 - [52] Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989;244:359–62.
 - [53] Rose TM. CODEHOP-mediated PCR – a powerful technique for the identification and characterization of viral genomes. *Virol J* 2005;2:20.
 - [54] Allander T, Emerson SU, Engle RE, Purcell RH, Bukh J. A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. *PNAS* 2001;98:11609–14.
 - [55] Belák S. Molecular diagnosis of viral diseases, present trends and future aspects. A view from OIE collaborating centre for the application of polymerase chain reaction methods for diagnosis of viral diseases in veterinary medicine. *Vaccine* 2007;25:5444–52.
 - [56] Hakhverdyan M, Rasmussen T, Thoren P, Uttenthal Å, Belák S. Development of a real-time PCR assay based on primer-probe energy transfer for the detection of swine vesicular disease virus. *Arch Virol* 2006;151:2365–76.
 - [57] Barfoed AM, Østergaard E, Frandsen PL, Nielsen EB, Sandberg E, Rasmussen TB. Development of a primer-probe energy transfer based real-time PCR for detection of Marek's disease virus. *J Virol Meth* 2010;165:21–6.
 - [58] Gustafsdóttir SM, Nordengrahn A, Fredriksson S, Wallgren P, Rivera E, Schallmeier E, et al. Detection of individual microbial pathogens by proximity ligation. *Clin Chem* 2006;52(6):1152–60.
 - [59] Dodet B, Hesselink W, Jungback C, Lechenet J, Pastoret PP, Vannier P, et al. Viral safety and extraneous agents testing for veterinary vaccines. *Biologicals* 2010;38:326–31.
 - [60] Lasmézas Cl, Comoy E, Hawkins S, Herzog C, Mouthon F, Konold T, et al. Risk of oral infection with bovine spongiform encephalopathy agent in primates. *Lancet* 2005;365:781–3.
 - [61] Wang D, Coscoy L, Zylberberg M, Avila PC, Boushey HA, Ganem D, et al. Microarray-based detection and genotyping of viral pathogens. *PNAS* 2002;99:15687–92.
 - [62] Erlandsson L, Rosenstjerne MW, McLoughlin K, Jaing C, Fomsgaard A. The microbial detection array combined with random Phi29-amplification used as

- a diagnostic tool for virus detection in clinical samples. *PLoS One* 2011;6:e22631.
- [63] Erlandsson L, Nielsen LP, Fomsgaard A. Amp-PCR: combining a random unbiased Phi29-amplification with a specific real-time PCR, performed in one tube to increase PCR sensitivity. *PLoS One* 2010;5:e15719.
- [64] CVMP/EMA: table of extraneous agents to be tested for in relation to the general and species specific guidelines on production and control of mammalian veterinary vaccines, <http://www.emea.europa.eu/pdfs/vet/vetguidelines/7blm10a.pdf>; 1994.
- [65] Dellepiane N, Griffiths E, Milstien JB. New challenges in assuring vaccine quality. *Bull WHO* 2000;78(2):155–62.
- [66] Weissmahr RN, Schüpbach J, Böni J. Reverse transcriptase activity in chicken embryo fibroblast culture supernatants is associated with particles containing endogenous avian retrovirus EAV-0 RNA. *J Virol* 1997;71(4):3005–12.