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Testing for viral contaminants of veterinary vaccines in Hungary

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ABSTRACT

The safety of veterinary vaccines is of paramount importance and it is significantly jeopardised by extraneous agents such as bacteria, mycoplasma, Chlamydia and viruses. Several critical steps of vaccine manufacture involve a potential risk of viral contamination. Viruses, as extraneous agents, can be divided into two main groups. Group 1 agents, such as Pestivirus, chicken anaemia virus (CAV), and egg drop syndrome virus (EDSV) are well-known to manufacturers and authorities. Compendial detection methods, clear guidelines and legislation have been established to minimise the risk of contamination with these agents. Contrary to group 1, group 2 agents like Torque Teno virus (TTV) or RD114, a replication-competent feline γ-retrovirus, have only recently been recognised and their role as contaminants needs further investigation.

Randomly selected veterinary vaccines used between 1992 and 2009 were tested by nucleic acid amplification for CAV, EDSV, and TTV. Pestivirus contamination was examined in 33 vaccines used between 1996 and 2006 and a further 27 vaccines used between 2007 and 2009 based on random selection of these vaccines. In addition to random tests done on vaccines used from 2007 on, 12 batches of live Aujeszky’s disease vaccines submitted to our laboratory for Official Control Authority Batch Release (OCABR) were also tested for Pestivirus.

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1. Introduction

Since Edward Jenner’s discovery of smallpox vaccine, which opened a new chapter in the combat against microbial pathogens, vaccination has represented the most feasible and cost effective way to prevent, control and eradicate infectious diseases [1]. Veterinary vaccinology is considered as a key factor in improving animal welfare, decreasing the cost of producing food animals and reducing the incidence of zoonoses [2].

Vaccinology has become a profoundly complex and multidisciplinary science, which includes immunology, microbiology, molecular biology, biochemistry and statistical sciences. Regulation, legislation and ethics play an important role as well. In parallel to the complexity of vaccinology, the international veterinary vaccine market has grown to US$3.1 billion.

Due to their biological nature, veterinary vaccines must meet numerous strict quality, efficacy and safety criteria. Vaccine safety is of paramount importance, and it can be significantly jeopardised by extraneous agents such as bacteria, mycoplasma, Chlamydia and viruses. The criteria for veterinary vaccines are well regulated by several bodies and testing requirements have been established for potency, efficacy, safety and purity [3]. The solid legal basis of veterinary vaccine control includes national and European Union (EU) legislation, guidelines from the Committee for Medicinal Products for Veterinary Use (CVMP), European Pharmacopoeia (Ph. Eur.) monographs and the World Organisation for Animal Health (OIE) Manual [4] (Fig. 1). Manufacturers are obliged to follow appropriate production procedures, under good manufacturing practices (GMP), and control starting materials, master seed virus (MSV), working seed virus (WSV), master cell stock (MCS), final products and production procedures (in-process control).

Beyond the manufacturers, competent authorities contribute through the authorisation and inspection of manufacturing sites, the assessment of registration dossiers of certain vaccines and the control of finished products. While these efforts minimise the risk of contamination, vaccine contamination cannot be excluded. This is clearly underlined by a few examples such as Pestivirus contamination of live vaccines for human use [5] or the presence of Newcastle disease virus (NDV) vaccine strains in different live poultry vaccines [6]. Nevertheless, the list of extraneous agents is growing with new members such as RD114 virus and Torque Teno virus (TTV). RD114 was first detected by Okada et al. [7] in a feline Parvovirus vaccine, while 6 out of 26 swine vaccines tested positive for TTV according to a recent study [8].
The present article summarises our experience, as a competent authority, in testing selected veterinary vaccines for extraneous agents.

2. Methods

2.1. Vaccines tested

Between 1996 and 2006, 33 vaccines against porcine reproductive respiratory syndrome (PRRS), infectious bovine rhinotracheitis (IBR), Aujeszky's disease, myxomatosis, equine influenza (EI), feline rhinotracheitis, feline panleukopenia, feline calicivirus and canine parvovirus produced by different companies were randomly chosen and tested for Pestivirus. Since 2007, 12 batches of live Aujeszky's disease vaccines have been tested for Pestivirus by reverse transcriptase-polymerase chain reaction (RT-PCR), in the framework of the Official Control Authority Batch Release (OCABR). In addition to OCABR testing, random tests were carried out on 27 batches of seven different vaccines against porcine parvovirus, swine erysipelas, IBR, PRRS, canine parvovirus and feline panleukopenia used in Hungary between 2007 and 2009. In addition, 27 poultry vaccines, from eight different manufacturers, used in Hungary between 1996 and 2009 were randomly selected and examined by PCR for the presence of chicken anaemia virus (CAV) and egg drop syndrome virus (EDSV). A total of 35 different vaccines, including all poultry vaccines mentioned above, were tested for TTV (Table 1). Only one PRRS vaccine and two batches of an Aujeszky's disease vaccine sent to OCABR and tested for Pestivirus were tested for TTV, due to the random selection.

2.2. Animal test

Three, two-month-old Kahyb breed pigs (Hungarian landrace hybrid), weighing approximately 12 kg were purchased from a commercial breeding farm. Two animals were vaccinated with an Aujeszky's disease vaccine sent to OCABR and tested for Pestivirus were tested for TTV, due to the random selection.

Table 1

| Tests for extraneous Torque teno virus in veterinary vaccines, 1991–2009. |
|-----------------|-----------------|-----|
| Viral content   | Number of tests | TTV positive |
| CPV            | 12              | 0   |
| ADV            | 1               | 0   |
| PRRS           | 1               | 1   |
| CCV            | 1               | 1   |
| FPV            | 3               | 2   |
| BCV            | 1               | 0   |
| NDV            | 13              | 0   |
| CAV            | 1               | 0   |
| APV            | 1               | 0   |
| GPV            | 1               | 0   |
| Total          | 35              | 15  |

Virus abbreviations: CPV, canine parvovirus; ADV, Aujeszky's disease virus; PRRS, porcine reproduction and respiratory virus; CCV, canine coronavirus; FPV, feline panleukopenia virus; BCV, bovine coronavirus; NDV, Newcastle disease virus; CAV, chicken anaemia virus; APV, avina polyomavirus; GPV, goose parovirus.
animal was used as a control. On day 70, blood was taken and tested by virus neutralisation assay.

2.3. Virus neutralisation assay

The neutralising antibody activity of sera obtained from vaccinated pigs was determined in a 96-well bottomed microtitre plate (DiaLab Ltd, Hungary) using baby hamster kidney (BHK) cells. Wells were observed for cytopathic effect and the neutralisation titre of the serum was expressed as the reciprocal of the final dilution of serum that neutralised 50% of virus activity.

2.4. PCR

2.4.1. Pestivirus PCR

To detect Pestivirus strains, the thermoprofile and primers published by Vlček et al. [9] were used. RNA was extracted by Trizol™ (Invitrogen) according to manufacturer’s instructions. The cDNA was synthesized in 25 µl containing 8 µl diethylpyrocarbonate (DEPC)-treated water, 5 µl 5× RT buffer, 0.5 µl each of 10 mM dNTP, 0.02 U random primer (Promega), 1 U Rnasine (Promega), 10 µl M-MLV-RT (Promega) enzyme and 5 µl RNA. The PCR amplification reaction was carried out in 50 µl containing 0.5 µl of each 10 mM dNTP (Promega), 15 pmol of each primer, 5 µl 10× PCR buffer (100 mM Tris–HCl, pH 9.0, 500 mM KCl and 1 mg/ml bovine serum albumin (BSA)), 2 mM MgCl₂ (50 mM), 1 U Taq polymerase (Promega) and 3 µl cDNA.

2.4.2. CAV and EDSV PCR

CAV PCR was carried out using the primers and thermoprofile published by Tham and Stanislawek [10] in a modified reaction mixture comprising 5 µl 10× PCR buffer (100 mM Tris–HCl, pH 9.0, 500 mM KCl and 1 mg/ml BSA), 1 µl MgCl₂ (50 mM), 0.5 µl of each dNTP (10 mM each, Pharmacia), 20 pmol of each primer, 2 U Taq polymerase (Invitrogen), and 5 µl DNA with ddH₂O added up to a total volume of 50 µl.

EDSVR PCR was carried out according to Xie et al. [11], with a modified reaction mixture optimised for vaccine amplification directly from the vial. The reaction mixture contained 5 µl 10× PCR buffer (100 mM Tris–HCl, pH 9.0, 500 mM KCl and 1 mg/ml BSA), 3 µl 50 mM MgCl₂, 0.5 µl of each dNTP (10 mM each, Pharmacia), 20 pmol of each primer, 2 U Taq polymerase (Invitrogen), and 5 µl DNA with ddH₂O added up to a total volume of 50 µl DNA for CAV and EDSVR PCR was extracted by Trizol™ (Invitrogen) according to the manufacturer’s instructions.

2.4.3. TTV PCR

A 230 bp ampiclon was obtained using a nested primer set and thermoprofile published by Kekarainen et al. [12]. A modified reaction mixture was applied containing 5 µl 10× PCR buffer (100 mM Tris–HCl, pH 9.0, 500 mM KCl and 1 mg/ml BSA), 3 µl 50 mM MgCl₂, 0.5 µl of each dNTP (10 mM each, Pharmacia), 20 pmol of each primer, 2 U Taq polymerase (Invitrogen), and 5 µl DNA with ddH₂O added up to a total volume of 50 µl DNA for TTV PCR was extracted by Trizol™ (Invitrogen) according to the manufacturer’s instructions.

2.5. Visualisation and sequence analysis

For visualisation, 8 µl of the PCR products were electrophoresed in 2% agarose gel. After electrophoresis, the gels were stained in ethidium bromide and viewed under ultraviolet light. The PCR products were sequenced by Biomi Ltd., Hungary, and the nucleotide and deduced amino acid sequences were aligned with the aid of BioEdit 7.0.9.0. software [13] using the Clustal method.

Table 2

| Results of the test | What should be done |
|---------------------|---------------------|
| PCR is negative     | No further tests. Product considered as non-contaminated. |
| PCR is negative or in vitro test is negative | No further test needed at this stage (unless justified: not optimal conditions for carrying out the in vitro test, see above). Product considered as non-contaminated. |
| In vitro test is positive | No need for in vivo test. Product considered as contaminated even if the PCR is negative. |
| PCR+/in vitro test cannot be carried out | The in vivo test is performed (this is done in the case of risk analysis for interference, under the BVD eradication programme) to verify the possibility of a contamination. |
| PCR+/in vivo test + | Product considered as contaminated. |

3. Results

3.1. Tests for Pestivirus

The vaccines tested proved to be free of Pestivirus except for one Aujeszky’s disease vaccine. In order to ascertain whether or not the positive result obtained by RT-PCR was due to laboratory contamination, the RT-PCR tests were repeated; the same result was obtained. As a further step, a new vial of the given vaccine was reconstituted and two susceptible pigs were vaccinated and observed together with one control animal. The sera of susceptible animals were used for virus neutralisation assay to test the titre of neutralisation antibodies induced by Pestivirus contamination of the vaccine, but no neutralisation activity was found.

3.2. Tests for TTV

PCR detected TTV nucleic acid in 5 poultry vaccines and 10 mammalian vaccines. All five positive poultry vaccines were live NDV vaccines with expiry dates ranging from 1997 to 2004. Out of the 10 TTV positive mammalian vaccines, 6 were live CPV vaccines including an archive vaccine, whose production was cancelled. One live PRRS vaccine and two feline panleukopenia virus vaccines were also positive. All vaccines were re-tested three times, confirming the positive TTV results.

The presence of TTV in these vaccines was confirmed by direct sequencing and revealed that the contaminant virus belongs to Pestivirus contamination of the vaccine, but no neutralisation activity was found.

3.3. Tests for CAV and EDSV

PCR testing was done on 27 poultry vaccines, used in Hungary, from eight different manufacturers. All vaccines tested proved to be negative for both CAV and EDSV.

4. Conclusions

Viruses as extraneous agents form two well-distinguishable groups. Group 1 agents, including Pestivirus, chicken anaemia virus (CAV) and egg drop syndrome virus (EDSV) are well known to manufacturers and the competent authorities. Compendial detection methods, clear guidelines and legislation have been established to minimise the risk of contamination. Contrary to the well-known Group 1 agents, Group 2 contains new potential contaminants, such as TTV and/or RD114 virus, recently found to be present
in vaccines. These new contaminant agents have mainly been detected by academic research groups using nucleic acid amplification tests (NAT), underlining the key role of research both in hunting for new viruses and the improvement and evaluation of new, better methods for detection.

Starting materials of animal origin, including bovine serum, SPF eggs and/or different tissues, such as CrFK are essential ingredients used in the production of many immunological veterinary medicinal products, but they also constitute one of the major sources of contamination. One of the specific risks associated with the use of bovine serum is the contamination of finished vaccine with bovine viral diarrhea virus (BVDV), while CAV and ESDV pose a similar threat to poultry vaccines via contaminated eggs. A potential source of RD114 contamination is the use of endogenous retrovirus susceptible cell lines, for instance CrFK [14], which is widely used to produce dog vaccines, especially against canine Coronavirus and Parvovirus. MTV may pose a threat to the safety of vaccines via contaminated bovine serum and contaminated tissues of bovine or porcine origin. On account of the wide species distribution of TTV, avian vaccines may be also affected by MTV contamination.

The significance of these agents may be underestimated at present. Increased expression of RD114 viral mRNA levels has been detected in feline sarcomas and lymphomas in domestic cats, but tumours have been found only with concurrent infection with another feline retrovirus [15]. There is no evidence to date that RD114 virus is involved in the development of fibrosarcoma in its natural hosts, however, a number of endogenous retroviruses can cause tumours [16–18] and the absence of retroviral oncogenes in an endogenous retrovirus does not exclude neoplastic potential; the viral LTR being may enhance expression of adjacent cellular genes, for instance proto-oncogenes. Tumour development can also depend on the proviral integration site.

There is growing evidence for the association of MTV with specific diseases such as post-weaning multisystemic wasting syndrome (PMWS) in swine, and rhinitis, asthma, hepatic disease, pancreatic cancer and idiopathic inflammatory myopathies in humans [19–25]. It should be emphasised that MTV has the ability to be transmitted vertically in humans and swine [26,27].

RD114, MTV and any other ‘new’ extraneous agents unequivocally need more consideration. Investigation of the viral cycle, prevalence studies and development of more efficient methods for detection are needed, as well as a regulatory approach to address these new contaminant agents.

The interpretation of positive PCR results is complex and often requires further testing, considering the fact that NAT detects nucleic acid, and not infectivity, and to rule out false positives. In the case of Pestivirus contamination, the CVMP of the European Medicines Agency (EMEA) issued a guideline [28] on how to interpret positive PCR results and what measures should be taken (Table 2). According to this guideline, positive PCR tests should be confirmed by in vivo testing to determine whether the result was due to a genomic fragment of BVDV, or a classical swine fever virus (CSFV), or live, intact virion.

In the case of positive PCR results for RD114 and/or MTV, a case-by-case approach is needed to interpret the results. The animal test is clearly hampered by the fact that RD114 is apathogenic in its natural hosts; MTV does not cause discernable symptoms either.

In summary, this study found that MTV was present in many vaccines including – surprisingly – avian vaccines. The presence of any extraneous agent may have a significant impact on the safety of the vaccine. However, further in-depth analysis is needed for group 2 extraneous agents to clarify the potential effect of these viruses on vaccine quality and safety. Standardised protocols on how to detect them in vials and clear guidelines for authorities and manufacturers on how to respond to their presence in vaccine starting materials or finished products are needed.

References

[1] Andre FE. Vaccinology: past achievements, present roadblocks and future promises. Vaccine 2003;2:593–5.
[2] Pastoret PP. Veterinary vaccinology. Comptes Rendus de l’Academie des Sciences – Series III – Sciences de la Vie 1999;322:967–72.
[3] Thornton DH. Quality control of vaccines. In: Alexander DJ, editor. Newcastle disease. Boston: Kluwer; 1988. p. 347–65.
[4] Tests for sterility and vaccine contamination of biological materials. In: Manual of standards for diagnostic tests and vaccines. 6th ed. Paris: Office International des Epizooties (OIE); 2008. p. 105–15.
[5] Giangaspero M, Vacca G, Harasawa R, Butterlin 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.
[6] Jorgensen PH, Handberg KJ, Ahrens P, Manwell 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.
[7] Okada M, Shojima T, Baba K, Ishikawa M, Miyazawa T. Expression of RD114 virus and its receptor in feline cell lines: potential risk of contamination of live attenuated vaccines by RD114 virus. In: 113th congress of Japan Society for Veterinary Medical Science; 2007.
[8] Kekarainen T, Martinez-Guinó I, Segales J. Swine Torque Teno virus detection in pig commercial vaccines, enzymes for laboratory use and human drugs containing components of porcine origin. J Gen Virol 2009;90(Pt 3):548–53.
[9] Vílcek 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.
[10] Tham KM, Stanislawek WL. Polymerase chain reaction amplification for direct detection of chicken anemia virus DNA in tissues and sera. Avian Dis 1992;36(4):1090–6.
[11] Xie Z, Fadl AA, Girshick T, Khan ML. Amplification of avian adenovirus by polymerase chain reaction. Avian Dis 2000;43:98–105.
[12] Kekarainen T, Sihla M, Segales J. Prevalence of swine Torque Teno virus in postweaning multisystemic wasting syndrome (PMWS)-affected and non-PMWS-affected pigs in Spain. J Gen Virol 2006;87:833–7.
[13] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 1999;41:95–8.
[14] Baumann JG, Gunzburg WH, Salmons B. CrFK feline kidney cells produce an RD114-like endogenous virus that can package murine leukemia virus-based vectors. Virology; 1998:7885–7.
[15] Niman HL, Stephensson JR, Gardner MB, Roy-Burman P. RD-114 and feline leukaemia virus genome expression in natural lymphomas of domestic cats. Nature 1977;268:5600):357–60.
[16] Bannert N, Kurth R. Retroelements and the human genome: new perspectives on an old relation. Proc Natl Acad Sci USA 2004;101(Suppl. 2):14572–9.
[17] Kasirga E, Sanlidag T, Akcali S, Keskin S, Aktas E, Karakoc Z, et al. Association of Torque Teno virus with rhinitis in a newborn. Clin Infect Dis 2003;36:128–9.
[18] Lower R, Lower J, Kurth R. The viruses in all of us: characteristics and biological significance of human endogenous retrovirus sequences. Proc Natl Acad Sci USA 1996;93(11):5777–84.
[19] Weiss RA. The discovery of endogenous retroviruses. Retrovirology 2008;3:57.
[20] Tomaszewicz K, Charrel RN, De Micco P, de Lamballe X. Association of MTV virus primary infection with rhinitis in a newborn. Clin Infect Dis 2003;36:128–9.
[21] Gergely P, Peri A, Poor G. Possible pathogenic nature of the recently discovered TV virus: does it play a role in autoimmune rheumatic diseases? Autoimmun Rev 2006;5:5–9.
[22] Kasirga E, Sanildag T, Akcali S, Keskis M, Akkoc Z, et al. Clinical significance of TV virus infection in children with chronic hepatitis B. Pediatr Int 2005;47:300–4.
[23] Pifferi M, Maggi F, Andreoli E, Lanini L, Marco ED, Forani C, et al. Associations between nasal Torque Teno virus load and spirometric indices in children with asthma. J Infect Dis 2005;192(7):1141–8.
[24] Tomaszewicz K, Modrzewska R, Lyczak A, Pola-Dawczewicz M, Rajtar B. TT virus (TVT) – etiologic agent of acute hepatitis? Ann Univ Mariae Curie Sklodowska Med 2004;59(2):539–42.
[25] Tomaszewicz K, Modrzewska R, Lyczak A, Krawczyk G. TT virus infection and pancreatic cancer: relationship or accidental coexistence. World J Gastroenterol 2005;11(18):4847–8.
[26] Gergely P, Peri A, Poor G. Possible pathogenic nature of the recently discovered TV virus: does it play a role in autoimmune rheumatic diseases? Autoimmun Rev 2006;5:5–9.
[27] Kasirga E, Sanildag T, Akcali S, Keskin S, Aktas E, Karakoc Z, et al. Clinical significance of TV virus infection in children with chronic hepatitis B. Pediatr Int 2005;47:300–4.
[28] Pifferi M, Maggi F, Andreoli E, Lanini L, Marco ED, Forani C, et al. Associations between nasal Torque Teno virus load and spirometric indices in children with asthma. J Infect Dis 2005;192(7):1141–8.