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Assessment report for Art 5(3) procedure:Detection by a highly sensitive new PCR technique of genomic fragments from endogenous and adventitious viral agents in live attenuated vaccines

Procedure number: EMEA/H/A-5(3)/1269

Assessment Report as adopted by the CHMP with all information of a commercially confidential nature deleted.

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1. Background information on the procedure

The European Medicines Agency (EMA) was made aware of a publication of an article[1] which presented new information regarding the possible presence of nucleic acid sequences from endogenous[2] and adventitious[3] viral agents in a few batches of different live attenuated viral vaccines tested by a new analytical technique. Whilst supporting the current record of safety of vaccines, this new advanced analytical metagenomics approach (polymerase chain reaction (PCR) combined with pyrosequencing) was used by a team of researchers in the United States (US) with the aim to determine whether low levels of nucleic acids e.g. originating from adventitious viruses "were introduced during the attenuation or manufacture of eight live viral vaccines".

Some batches of live attenuated vaccines tested by the research team had tested positive for either sequences of endogenous avian leukosis virus (ALV), or endogenous simian retrovirus (SRV), or porcine circovirus 1 (PCV-1). In view of the above, the Executive Director of the EMA presented on 13 April 2010 a request for a CHMP (Committee for Medicinal Products for Human Use) opinion under Article 5(3) of Regulation EC (No) 726/2004.

A CHMP opinion was sought on the following:

1. Whilst the presence of endogenous ALV and SRV DNA fragments is potentially linked to the cell lines used for the manufacture of the individual vaccines, the finding of PCV-1 DNA pointed towards the presence of non-endogenous viral DNA. The Committee is therefore asked to assess if there is any potential public health concern arising from all the findings described in the article.

2. It is understood that as more hi-tech analytical methods become available, new findings may reveal in medicinal products presence of substances at very low concentrations and/or hard to detect that were thus far not picked up because of limitations of the analytical instruments/methods declared and authorised in the marketing authorisation dossier. Specifically, the Committee is asked to indicate if the new test method described above applied to identify viral genomic nucleic acid fragments should be considered, if validated, for the development/qualification and/or routine testing of biological medicinal products, and particularly live attenuated vaccines. Recognising the limitations of this novel method, and in order to conclude on the presence or absence of specific risks, the Committee is also asked to consider whether additional tests would be needed to conclude on the presence of replication competent endogenous and adventitious viral agents.

3. The Committee should indicate if there is a need to update existing guidance related to the testing and elimination of such substances in the context of development and/or testing of live attenuated vaccines.

4. Depending on the outcome of these investigations, the Committee should consider the need for appropriate guidance for other vaccines and other biological products.

To further aid the CHMP in reaching its opinion an *ad-hoc* expert meeting was convened on 13 September 2010 at the EMA. This assessment report considers the views of this expert meeting, alongside the available data from published literature, including available guidelines. The Biologics Working Party (BWP) was consulted for the assessment of this procedure, as appropriate. Interaction with the European Pharmacopeia and international partners, including the Food and Drug Administration (FDA) and World Health Organisation (WHO) was sought.

[1] Viral Nucleic Acids in Live-Attenuated Vaccines: Detection of Minority Variants and an Adventitious Virus. Victoria JG, Wang C, Jones MS, Jaing C, McLoughlin K, Gardner S and Delwart EL. *J Virol. 2010 Jun;84(12).* http://jvi.asm.org/cgi/content/short/84/12/6033

[2] Endogenous agents are those whose genome is present in an integrated form in a cell substrate by heredity. Endogenous viral agents are considered to be originated from an ancient viral infection of the hosts from which the cell substrates/lines are derived, passed on from generation to generation and now remaining in their genome.

[3] Adventitious agents are microorganisms that have been unintentionally introduced into the manufacturing process of a biological product; they include bacteria, fungi, mycoplasmas, rickettsia, protozoa, parasites, TSE agents, and viruses.

2. Scientific discussion

2.1. Introduction

On 13 April 2010 the Executive Director of the EMA asked the CHMP, in accordance with Article 5(3) of Council Regulation (EC) No 726/2004, to give an opinion on questions regarding any potential public health concern arising from the detection of genomic fragments from endogenous and adventitious viral agents in live attenuated vaccines, if the new techniques described in a published article used to detect these agents should be considered for the development/qualification and/or routine testing of biological medicinal products, and the need to consider revising available guidance.

Live-attenuated vaccines are those produced by attenuating or weakening the virus to a point that it is no longer able to cause disease. The purified attenuated live virus is then used in the vaccine. The manufacturing of live attenuated vaccines may include different animal substrates and/or be derived from different cell lines. Although the manufacturing process for live attenuated vaccines does not contribute to the inactivation/elimination of adventitious agents (the process itself not including specific inactivation steps), the cell substrates, viral seeds, media components and components used in production and purification of live attenuated vaccines are regulated through a variety of tests which may vary in accordance with the product being tested to ensure its quality. These rules also apply to other vaccines and other biological medicinal products.

Methods for the detection of endogenous and adventitious virus include a combination of strategies at different stages in the development and production process. Several novel analytical screening systems that can be used for the detection of adventitious agents have been developed. These include but may not be limited to:

- oligonucleotide arrays to detect viral genomes or mRNA,
- PCR (degenerate, multiplex, etc.) followed by mass spectrometric analysis of the amplicon(s),
- nanotechnology, consisting of direct detection of nucleic acids using labelled probes,
- massively parallel (deep) sequencing of randomly amplified nucleic acids (MP-sequencing)[4].

The workflow of MP-sequencing may vary depending on available technical platforms but generally includes the generation of a library of DNA fragments, its amplification, sequencing and analysis using bio-informatics tools. Different platforms are currently available, offering different advantages, which may include read length, number of reads, throughput and costs. Since this technology is still under development, new alternatives are to be expected in the future. Whereas the number of different platforms is still limited, the range of bio-informatics tools and related software is extremely wide, and many algorithms are in development. Moreover, the sequence databases to be searched for matches with the sequencing reads generated by the new techniques are evolving and their size/quality could impact the validity of the outcome.
Nevertheless, MP-sequencing and related software are potentially valuable tools in the development/qualification of biologics and the detection of adventitious viruses, mycoplasma and bacteria as well as integrated and latent viruses or cellular transcripts specifically expressed in infected cells such as interferon messenger RNA. As compared to most other adventitious agent detection methods, MP-sequencing has similarities to PCR but it can screen for the presence of genetic sequences of unknown microbial and/or viral contaminants, in contrast to classical PCR which targets specific known sequences.

However, MP-sequencing used as an analytical procedure has not yet been validated. Although it can, to some extent, display semiquantitative results, this methodology can essentially be presently categorised as a limit test for the control of impurities. Accordingly, the specificity and the sensitivity/detection limit are essential validation characteristics to be dealt with. It is foreseen that this method can be tested for these validation characteristics when applied to the detection of known targets but this could take several years. As for unknown sequences, validation is most likely not

[4] In the past years, new technologies for the determination of nucleotide sequences have been developed. Collectively they are known as massively parallel (deep) sequencing. This terminology derives from their ability to determine a huge number of individual sequences, in parallel, within one single reaction vessel. The number of nucleotides that can be sequenced with this technology is of the order 106 in one overnight run. One further advantage of the new technologies is their ability to sequence virtually all nucleic acid molecules within a sample without prior knowledge of the nature of the sequences within the sample. Application of these technologies is the basis of the so-called metagenomics approach.

feasible. The specificity of the method is given by the read sequence itself and dependent upon the parameters of the bio-informatic analysis. The sensitivity relies on both the technology and the bio-informatics. This could be assessed by both *in vitro* and *in silico* spiking experiments. In this regard, recovering of known amounts of specific target nucleic acids spiked into a sample could possibly identify the target thresholds. Similarly, inclusion of artificial electronic sequences in the reads set could give an insight on the ability of the bio-informatic analysis for detecting rare reads. In contrast, the true limit of detection of an unknown contaminant remains a difficulty, as this is linked to the claim of the detection of a signal or the claim that there is no contaminant present.

Metagenomics' hits (or absence thereof) need to be confirmed by other means such as specific NAT (nucleic acid based tests) and/or conventional virological or microbiological testing. The relevance of metagenomic signals and whether or not they constitute a risk should be addressed on a case by case basis as it is depending on the nature/identity of the newly detected/identified contaminant. Where positive signals are generated, these should be confirmed; it is expected that false negative results could arise from matrix interference, inappropriate bio-informatic algorithms used or from insufficient sequence databases coverage. Detection of low level viral contamination could fail due to insufficient sensitivity. False positive results (in the strict sense analytical specificity) are not relevant as this technique does not detect known sequences. False positive results could derive from cross-contamination, cellular amplicons erroneously interpreted as virus sequences, or viral sequences harbouring cellular sequences (bio-informatics issues).

Although validation of MP-sequencing could eventually be achieved, currently its standardisation, although highly desirable, is not deemed possible, essentially because not only the technology but above all, the bio-informatics is diverse and still evolving. The use of relevant nucleotide sequence databases and the validation of certain search parameters (bio-informatics/algorithms) are required. The databases need to be proofread and regularly updated. Finally, the impact of the sample preparation, amplification, sequence reaction, and bio-informatic algorithms used is of extreme importance on the reliability of the search results.
Test validation would need to cover the broad variety of biochemical and bio-informatical approaches. It is understood that this might be the scope of future guidance.

2.2. Discussion

2.2.1 Question 1

Whilst the presence of endogenous ALV and SRV DNA fragments is potentially linked to the cell lines used for the manufacture of the individual vaccines, the finding of PCV-1 DNA pointed towards the presence of non-endogenous viral DNA. The Committee is therefore asked to assess if there is any potential public health concern arising from all the findings described in the article.

The detection of sequences of endogenous virus in the manufacturing of vaccines as reported in the said article was not an unexpected finding. Endogenous viral sequences are present due to heredity and the sequences from ALV or SRV as referred in the published article do not raise any concerns.

The unexpected presence of non-endogenous viral fragments of PCV detected using a new non-specific analytical detection method (metagenomics analysis, with pyrosequencing) which was not part of the approved control method used was considered in the appropriate regulatory framework[5]. This discussion leads to the conclusion that the presence of PCV in the identified rotavirus vaccines did not affect their benefit risk balance. It is noted that as a sample/product is retrospectively found positive via new analytical methods, including metagenomic analysis, it should lead to a risk assessment exercise in which actual safety data collected during clinical trial and/or pharmacovigilance should play a central role for evaluating the benefit-risk ratio. The use of new technologies may lead to the detection of unexpected findings; however a flexible approach based on a proper benefit-risk assessment and appropriate regulatory processes would likely need to be applied on a case-by-case basis.

[5] Article 20 review procedures were triggered for both centrally authorised live attenuated rotavirus vaccines, Rotarix and RotaTeq. For details on these procedures, please refer to the relevant European public assessment reports published at www.ema.europa.eu.

Taken together all available data, and after intensive scientific investigation it is considered that the findings of the article in question do not give rise to a public health concern.

2.2.2 Question 2

It is understood that as more hi-tech analytical methods become available, new findings may reveal in medicinal products presence of substance at very low concentrations and/or hard to detect that were thus far not picked up because of limitations of the analytical instruments/methods declared and authorized in the marketing authorisation dossier. Specifically, the Committee is asked to indicate if the new test method described above applied to identify viral genomic nucleic acid fragments should be considered, if validated, for the development/qualification and/or routine testing of biological medicinal products, and particularly live attenuated vaccines. Recognizing the limitations of this novel method, and in order to conclude on the presence of absence of specific risks, the Committee is also asked to consider whether additional tests would be needed to conclude on the presence of replication competent endogenous and adventitious viral agents.

A literature review of transmission and contamination events in medicinal products revealed numerous examples from the last twenty years documenting that viral contaminations were occasionally discovered in starting materials, drug substance as well as drug products of a broad range of biological medicinal products. Those contaminations were very often discovered only after the products were licensed and released for use. The majority of such events were linked to human plasma and other starting materials of human or animal origin but also cell substrates or reagents for cell culture used for the production of viral vaccines were discovered as sources for contamination with viral adventitious agents. Technological development can sometimes be linked with new unexpected findings. For example, for plasma derived medicinal products, more sensitive detection techniques and virus inactivation techniques had to be introduced as patients receiving those products indeed manifested viral infections. It is questionable whether the novel "deep-sequencing approach" would have been suitable to avoid these cases due to the limited assay sensitivity of the novel approach with respect to specific pathogens. No signals for transmission of disease from adventitious viruses were ever observed with vaccines, even in the rare cases where viral vaccines were indeed contaminated with adventitious viral agents such as SV40 or SCMV as evidenced by sensitive infectivity assays introduced post-licensing.

It is understood that as new analytical methods become available, new findings may reveal in medicinal products the presence of substances at very low concentrations and/or hard to detect that were thus far not picked up. This may be a limitation inherent to the development of new techniques to detect and test for the unknown. However, the relevance and putative impact of nucleic acid presence, possibly detected at extremely low levels of concentration, is questionable and should be carefully addressed. It is acknowledged that the fact that PCV nucleic acids were now detected using metagenomics is not attributable to limitations of currently used instruments/methods. Indeed, standard PCR testing of the vaccines targeting PCV sequences gives a positive result, allowing its detection.

The metagenomics technique can however be suitable to identify adventitious DNA and RNA fragments that may not necessarily be detected by methods and techniques currently used by manufacturers and authorised for use. The conceptual advantage of this novel PCR approach is that it can, in principle, screen for the presence of unexpected or unknown microbial and/or viral contaminants, in contrast to classical PCR which targets only predefined specific known sequences. These techniques could be used for the qualification of cell lines, seed lots and materials used for their development. Testing of at least one bulk before purification could also be considered since it corresponds to the manufacturing stage where the contaminant is expected to be at the highest concentration. Prospective analyses may be preferred to retrospective reviews of available products with a satisfactory safety record. Testing of authorised products could also be considered for those displaying a higher risk, notably the live attenuated vaccines.

However, MP-sequencing as an analytical procedure has not yet been validated. It is foreseen that it can be assessed for specificity and sensitivity/limit of detection but this could take several years and could be particularly challenging for unknown and/or newly detected sequences. Whereas validation of MP-sequencing could eventually be achieved, at present its standardisation, may not be possible. The technology and the bio-informatics needed is diverse and still evolving. The use of relevant nucleotide sequence databases and the validation of certain search parameters (bio-informatics/algorithms) are

required. The databases need to be proofread and updated. Clearly, the impact of the sample preparation, amplification, sequence reaction, and bio-informatic algorithms used is of extreme importance on the reliability of the search results.

Additionally, metagenomics' hits (or absence thereof) need to be confirmed by other means such as specific NAT and/or conventional virological or microbiological testing. The relevance of metagenomic signals and whether or not they constitute a risk needs to be addressed on a case by case basis as it is depending on the nature/identity of the newly detected/identified contaminant.

Notwithstanding these facts, MP-sequencing and related software are potentially valuable tools in the development/qualification of biologics, including live attenuated vaccines, and the detection of adventitious viruses, mycoplasma and bacteria as well as integrated and latent viruses or cellular transcripts specifically expressed in infected cells such as interferon messenger RNA. This method has already been applied for addressing fermenter contaminations, characterising new vaccine cell substrates, raw materials and virus seeds, as well as testing of finished products. It remains however under development and guidance on its use may need to be considered.
Furthermore, although there is value in introducing this methodology, it is to be understood as a supplement to current standards of testing which cannot be replaced.

2.2.3 Question 3

The Committee should indicate if there is a need to update existing guidance related to the testing and elimination of such substances in the context of development and/or testing of live attenuated vaccines.

It is noted that apart from the *Position paper on viral safety of oral poliovirus vaccine* (OPV) (CPMP/BWP/972/98) essentially driven on simian primary cells SV40 contamination, there is neither CHMP nor ICH guidelines whose scope covers specifically the testing and elimination of viruses from live attenuated vaccines. The general guidance ICH Q5A(R1) (CPMP/ICH/294/95) *Derivation and Characterization of Cell Substrates Used For Production of Biotechnological/Biological Products* is very general, recommending: 'Virus testing of cell substrates should be designed to detect a wide spectrum of viruses by using appropriate screening tests and relevant specific tests, based on the cultivation history of the cell line, to detect possible contaminating viruses'. This guidance also refers to the WHO *Requirements for the Use of Animal Cells as in vitro Substrates for the Production of Biologicals*.

The Ph.Eur. method 2.6.16. *Tests for extraneous agents in viral vaccines for human use and general texts* 5.1.7. *Viral safety* and 5.2. 3. *Cell substrates for the production of vaccines for human use* deal with viral safety consideration of either cell substrates or virus seeds, but none of these documents are deemed appropriate to include requirements on metagenomic testing. Given the novelty and the absence of standardisation of metagenomic testing, including such testing in pharmacopoeias as compendial requirements is not recommended for the moment.

Some guidance documents (e.g. ICH Q5D: *Derivation and Characterization of Cell Substrates Used For Production of Biotechnological/Biological Products*) are applicable to the manufacture of products belonging to different class of risks (e.g. live attenuated vaccines as well as recombinant biotech proteins) but this makes their revision difficult; furthermore the inclusion of a "metagenomic" recommendation equally applicable to all products falling under the scope a same guideline, whatever their risk level does not seem appropriate at the moment. It should also be noted that the recommendations made in current guidance documents are not incompatible with the inclusion of new testing methods such as MP-sequencing. Finally, there are several guidance and compendial documents, issued from different institutions (CHMP/BWP, ICH, EDQM, WHO), that are related to the viral safety and the cell substrates.

Therefore, and considering all limitations regarding validation of MP-sequencing, more flexible guidance may be currently advisable, and a reflection paper could be developed to communicate the current status of discussions on the use of metagenomics in biologicals such as live attenuated vaccines. Additionally, since the most likely cause of the unexpected presence of PCV is porcine trypsin, reflection is given to the need for guidance on this reagent. Considering that qualification of a reagent is expected to be a simpler approach than qualification of medicinal products themselves, and that a stepwise approach would be advisable, implementation of a guideline on porcine trypsin appears as the most relevant and straightforward approach and should receive priority.

2.2.4 Question 4

Depending on the outcome of these investigations, the Committee should consider the need for appropriate guidance for other vaccines and other biological product.

Metagenomic testing may hold potential for an added value in the viral safety of the biological medicinal products, therefore it could be advisable to develop a position statement dedicated to the use and implementation of this new technology in e.g. prospective development of live vaccines. Medicinal products that do not have a viral reduction step in the manufacturing process present the greatest risk of transmitting a viral contaminant e.g. live viral vaccines, and ATMPs. Although MP-sequencing could eventually prove to be suitable for testing of live allogeneic cell therapy and live gene therapy medicinal products, the ATMPs display very distinctive features and, accordingly, they should be addressed separately. For other products that include viral inactivation and/or removal steps the risk is reduced e.g. inactivated vaccines, recombinant proteins, animal and human-derived medicinal products.

Therefore, the CHMP considered that a guideline of porcine trypsin is currently deemed appropriate and sufficient to ensure absence of PCV from live attenuated and inactivated viral vaccines as well as for other biological medicinal products for human use. There is no urgent need for an ad hoc implementation of guidance relevant to the detection of other potential viral contaminants in these products.

3. Conclusion

The CHMP was asked to give an opinion on questions regarding any potential public health concern arising from the detection of genomic fragments from endogenous and adventitious viral agents in live attenuated vaccines, if the new techniques described in a published article used to detect these agents should be considered for the development/qualification and/or routine testing of biological medicinal products, and the need to consider revising available guidance.

Based on all available data, the CHMP considered that the findings of these particular endogenous and adventitious viral sequences do not raise any public health concern.

The metagenomics approach (PCR combined with pyrosequencing) is defined as a step wise approach which includes sample selection (cellular genome (DNA), transcriptome (RNA), virus particle), amplification schemes (family specific primers, degenerate primers) and detection methods (mass spectrometry, microarray, high-throughput sequencing, among others). Its use for the development/qualification and/or routine testing of biological medicinal products appears as a valuable method for detection of a broad range of adventitious agents and could be considered as an additional tool for the prospective testing of biological medicinal products and/or their starting materials. This methodology could also be used retrospectively, for testing products already authorised. However, MP-sequencing sensitivity and specificity have not been clearly defined and this method is not currently validated rendering its use limited at present. Furthermore, any positive hit (or absence thereof) need to be confirmed by other means such as specific NAT and/or conventional virological or microbiological testing. Furthermore, each potential signal and its relevance would then need to be addressed on a case by case basis as it is depending on the nature/identity of the newly detected/identified agent.

It is apparent that animal-derived materials contain a wide range of adventitious viruses and therefore it is strongly recommended that all such materials are tested and treated appropriately for the inactivation and/or elimination of such agents. The presence of contaminants i.e. viable and non-viable cell substrates, non-infectious viral particles, and nucleic acid fragments should thus be controlled. Whilst there is guidance available, the need for additional guidance on porcine trypsin has been identified. In addition, the development of a reflection paper on metagenomic testing of live vaccines appears advisable. Medicinal products that do not have a viral reduction step in the manufacturing process present the greatest risk of transmitting a viral contaminant e.g. live viral vaccines. For products that include viral inactivation and/or removal steps the risk is reduced e.g. inactivated vaccines, recombinant proteins, animal and human-derived medicinal products. Any unexpected findings that might arise will need to be evaluated and appropriate regulatory processes for benefit risk balance assessment would need to be applied on a case by case basis.

Therefore, consideration should be given to guidance on live attenuated vaccines at the moment.

When validated, these new technologies could provide a useful additional screening method for adventitious viruses in cell banks, virus seeds and animal-derived materials. Although there may be value in introducing this methodology, it is to be understood as a supplement to current standards of testing which cannot be replaced.

The issues identified by the Committee are common to regulators worldwide. The CHMP has started a dialogue with other authorities, including the US Food and Drug Administration, the World Health Organisation and the European Directorate for the Quality of Medicines, to start working towards a common approach for the use of metagenomic testing in biological medicines. A common road map will be sought whenever possible.

4. Overall conclusions

The Committee considered the procedure under Article 5(3) of Regulation (EC) No 726/2004 for live attenuated vaccines initiated by the Executive Director of the European Medicines Agency.

The Committee considered relevant expertise, data available in the literature and guidelines in relation to the detection of endogenous and adventitious viral agents.

The Committee concluded that there is no public health concern arising from the detection of endogenous or adventitious viral DNA in some live attenuated vaccines.

The Committee concluded that a guideline on porcine trypsin should be developed.

The Committee concluded that a reflection paper on the use of metagenomics in live attenuated vaccines should be considered by the BWP.

The Committee noted that the BWP should interact with the EDQM and international partners, as applicable.

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