

Dossier EMA - NGS

Discussione dei risultati ottenuti dall'indagine
sulla qualità dei vaccini

Discussione dei risultati ottenuti dall'indagine sulla qualità dei vaccini

Di seguito si approfondiranno i risultati presentati dall'Associazione Corvelva, sull'analisi mediante LC-MS del contenuto del vaccino Priorix Tetra inviato ad Aifa ed EMA.

Priorix Tetra ¹

Riassunto delle caratteristiche del prodotto

RIASSUNTO DELLE CARATTERISTICHE DEL PRODOTTO

1. DENOMINAZIONE DEL MEDICINALE

Priorix Tetra - polvere e solvente per soluzione iniettabile
Vaccino (vivo) antimorbillo, antiparotite, antirosolia e antivaricella

2. COMPOSIZIONE QUALITATIVA E QUANTITATIVA

Dopo la ricostituzione, 1 dose (0,5 ml) contiene:

virus del morbillo ¹ ceppo Schwarz (vivo, attenuato)	non meno di 10 ^{5,0} CCID ₅₀ ³
virus della parotite ¹ ceppo RIT 4385, derivato dal ceppo Jeryl Lynn (vivo, attenuato)	non meno di 10 ^{4,5} CCID ₅₀ ³
virus della rosolia ² ceppo Wistar RA 27/3 (vivo, attenuato)	non meno di 10 ^{5,0} CCID ₅₀ ³
virus della varicella ² ceppo OKA (vivo, attenuato)	non meno di 10 ^{3,3} PFU ⁴

¹ coltivato in colture di cellule embrionali di pollo

² coltivato in cellule diploidi umane (MRC-5)

³ dose infettante la coltura cellulare al 50% (Cell Culture Infective Dose 50%)

⁴ unità formanti placca (Plaque Forming Units)

Questo vaccino contiene tracce di neomicina. Vedere paragrafo 4.3.

Eccipiente con effetto noto:

Il vaccino contiene 14 mg di sorbitolo.

Per l'elenco completo degli eccipienti vedere paragrafo 6.1.

3. FORMA FARMACEUTICA

Polvere e solvente per soluzione iniettabile.

Prima della ricostituzione, la polvere è una pastiglia di un colore che varia dal bianco al rosa chiaro e il solvente è un liquido chiaro e incolore.

6. INFORMAZIONI FARMACEUTICHE

6.1 Elenco degli eccipienti

Polvere:

Amminoacidi
Lattosio anidro
Mannitolo
Sorbitolo
Medium 199

Solvente:

Acqua per preparazioni iniettabili

¹ PRODUCT MONOGRAPH <https://ca.gsk.com/media/591336/priorix-tetra.pdf>

SPC – AIFA https://farmaci.agenziafarmaco.gov.it/aifa/servlet/PdfDownloadServlet?pdfFileName=footer_000200_038200_RCP.pdf&retry=0&sys=m0b1i3



DESCRIZIONE

PRIORIX-TETRA (vaccino combinato morbillo, parotite, rosolia e varicella, vivo, attenuato) è una preparazione mista liofilizzata del morbillo Schwarz attenuato, parotite RIT 4385 (derivato dal ceppo Jeryl Lynn), ceppo Wistar RA 27/3 e ceppi varicella Oka di virus.

Di seguito sono elencate le linee guida di riferimento per la produzione di Priorix Tetra:

- Annex I: List of the names, pharmaceutical forms, strengths of the medicinal products, routes of administration, marketing authorization holders in the member states
https://www.ema.europa.eu/en/documents/referral/monovalent-multivalent-measles-mumps-rubella/varicella-vaccines-article-31-referral-annex-i_en.pdf
- WHO TECHNICAL REPORT SERIES N. 840 1994 ANNEX 3: REQUIREMENT FOR MEASLES, MUMPS AND RUBELLA VACCINES AND COMBINES VACCINE (LIVE)
https://www.who.int/biologicals/publications/trs/areas/vaccines/mmr/WHO_TRS_840_A3.pdf?ua=1
- WHO TECHNICAL REPORT SERIES N. 848 1994 ANNEX 1: REQUIREMENT FOR VARIVELLA VACCINE (LIVE)
https://www.who.int/biologicals/publications/trs/areas/vaccines/varicella/WHO_TRS_848_A1.pdf?ua=1

Poiché Priorix tetra non è un vaccino autorizzato mediante procedura centralizzata, ma commercializzato in Italia mediante mutuo riconoscimento e l'AIFA non ha fornito ad oggi, alcuna informazione sul processo di produzione di Priorix tetra, viene preso come riferimento per produzione e specifiche, il vaccino MMRV ProQuad ² registrato da EMA.

2. ASPETTI SULLA QUALITÀ

Poiché AIFA non ha fornito informazioni sullo sviluppo e la produzione del ceppo di Schwartz, aggiungiamo qui di seguito alcuni documenti riguardanti lo sviluppo del ceppo:

ACTIVE SUBSTANCE – MEASLES

The Schwarz vaccine strain was generated in 1962 by additional passaging of the Edmonston strain in chick embryo fibroblast
[Am J Dis Child](#). 1962 Mar;103:386-9.

Preliminary tests of a highly attenuated measles vaccine.

[SCHWARZ AJ](#).

https://www.researchgate.net/publication/51212585_Genetic_Characterization_of_Measles_Vaccine_Strains

[J Infect Dis](#). 2011 Jul;204 Suppl 1:S533-48.

Genetic characterization of measles vaccine strains.

[Bankamp B1](#), [Takeda M](#), [Zhang Y](#), [Xu W](#), [Rota PA](#).

[Author information](#)

Abstract

The complete genomic sequences of 9 measles vaccine strains were compared with the sequence of the Edmonston wild-type virus. AIK-C, Moraten, Rubeovax, Schwarz, and Zagreb are vaccine strains of the Edmonston lineage, whereas CAM-70, Changchun-47, Leningrad-4 and Shanghai-191 were derived from 4 different wild-type isolates. Nucleotide substitutions were found in the noncoding regions of the genomes as well as in all coding regions, leading to deduced amino acid substitutions in all 8 viral proteins. Although the precise mechanisms involved in the attenuation of individual measles vaccines remain to be elucidated, in vitro assays of viral protein functions and recombinant viruses with defined genetic modifications have been used to characterize the differences between vaccine and wild-type strains. Although almost every protein contributes to an attenuated phenotype, substitutions affecting host cell tropism, virus assembly, and the ability to inhibit cellular antiviral defense mechanisms play an especially important role in attenuation.

[EPI News](#). 1980 Feb;2(1):6.

Live attenuated measles vaccine.

Abstract

² SCIENTIFIC DISCUSSION PROQUAD

https://www.ema.europa.eu/en/documents/scientific-discussion/proquad-epar-scientific-discussion_en.pdf

PIP:

This article reviews the history of measles vaccine development. Such a review is considered useful for understanding the relationship of the different brands of live attenuated measles vaccine available. In 1954, the measles virus was isolated from an 11-year old boy from the US, David Edmonston, and adapted and propagated on chick embryo tissue culture (CE). The CE adapted strain, known as Edmonston A, was too virulent for vaccine purposes. The strain was attenuated by means of further passages on CE fibroblasts, resulting in a 2nd generation attenuated virus designated as Edmonston B. Again, the strain was too virulent to be applied on a large scale. Laboratories continued to pass Edmonston B on CE until a 3rd generation of more attenuated strains was developed. These strains, which are known by different names and differ from each other in the number of times the parent strain was passed on CE, provide the seeds for the vaccines now commercially available. Among the trade names for live attenuated measles vaccine derived from the Edmonston B strain are Rimevax, Attenuvax, Rouvax, Lirugen, Morbilvax, Mevilin, and Moraten. At the same time, a USSR scientist developed a different parent seed known as Leningrad 16. The measles vaccines supplied through the World Health Organization's programme on Immunization in the Americas are prepared from seeds derived from Edmonston B.

Controls of materials and critical steps / process validation

The CEC (Chicken embryo cells) substrate used in the manufacture of measles vaccine bulk is tested according to Ph. Eur. requirements. Testing of the measles stock seed is consistent with the Ph. Eur., Section 2.6.16 and the monograph for Measles Vaccine (Live), with the exception of the virus identification test. Identity testing is instead performed post-clarification on the vaccine bulk, where antibody neutralization can be performed on a clarified bulk virus solution. Critical process parameters (CPPs), critical quality attributes (CQAs), and their specifications/acceptance criteria are based on historical process capability, current manufacturing specifications, and the specifications defined in the company's monovalent measles vaccine license. Process validation was both retrospective and prospective. Retrospective validation of measles vaccine was first used to determine acceptable ranges; a prospective validation of measles vaccine was then performed to demonstrate conformity of the processes to validation specifications. Within each manufacturing process step, goals, CPPs and CQAs were determined, along with appropriate specifications and acceptance criteria.

Characterisation and specifications

The complete nucleotide sequences for the Stock Seeds and a monovalent measles filled container vaccine lot have been determined. Nucleotide sequence alignment showed complete agreement.

Process-related impurities arising from the measles vaccine bulk manufacturing processes are classified as cell substrate or cell culture derived. Cell substrate derived impurities may include proteins derived from the host organism, such as CECs used as substrate for measles vaccine bulk production. Cell culture-derived impurities may include antibiotics (e.g., neomycin), serum, or other media components. Also low levels of particle-associated reverse transcriptase activity are found; however, no signal of infectious retrovirus could be detected. Since the measles process uses cell growth medium containing fetal bovine serum (FBS), measures have been taken to minimize the concentration of bovine serum proteins in the vaccine bulk. The concentration of bovine serum albumin (BSA) is used as a surrogate marker for other bovine serum proteins. Each measles final bulk is tested for BSA. Measles vaccine bulk is an unpurified product whose potency was measured through a biological assay for the active substance rather than through evaluation of integrity of physical form. Degradation products are neither identified nor quantified. Tests are performed at specified stages of vaccine bulk processing in order to confirm absence of extraneous agents, to verify potency and identity, and to provide a measure of quality and process consistency. Most assays performed on measles bulks are qualitative methods for which there are only two outcomes (growth or no growth, absence or presence, etc.). In many of these cases, the assay specifications are compendial. The parameters that were evaluated as part of the method validation for the assays have been provided for each analytical procedure. When applicable, the assay parameters addressed were specificity, interassay precision, limit of detection, limit of quantification, linearity, range, ruggedness, and robustness. Batch analysis results have been provided for HVF/HCF lots and dispensed bulk lots; all results met specifications.

ACTIVE SUBSTANCE - MUMPS

Seed lot system

The Jeryl Lynn strain of mumps virus was isolated from a throat washing specimen collected in 1963 from a clinical case of mumps (Jeryl Lynn) by Dr. M. R. Hilleman, Merck Research Laboratories, Merck & Co., Inc. Virus strain isolation was performed at the Merck West Point, Pennsylvania facility.

Manufacture of mumps harvested virus fluids (HVF) and redispensed bulk

CEC are planted in analogy to the process described for measles.

[Chicken embryo cells (CEC) as cell substrate

To prepare the cell substrate for virus propagation, eggs, sourced from a specific-pathogen-free (SPF) chicken flock, are incubated and prepared.

Manufacture of measles harvested virus fluids (HVF)

A virus propagator, a stainless steel tank, is planted with CEC suspension. The cells are infected with an appropriate volume of thawed measles stock seed, added to the seeding medium, stirred and incubated. The cell sheets are rinsed and refed several times and virus propagators are harvested. HVF is sampled for virus potency and sterility.

Manufacture of redispensed bulk

Harvests from one or more batches of HVF may be used to produce a single batch of measles vaccine bulk. The final bulk is dispensed in cans (dispensed bulk) and stored frozen. The dispensed bulk cans comprise a batch of drug substance. The dispensed bulk is thawed and used for filling or redispensed into aliquots appropriate for filling (redispensed bulk). Samples for QC are drawn from the appropriate different bulk stages.

Control cell Cultures and Harvest Control Fluids (HCF)

Uninfected harvested control fluids (HCF) are produced using the same cell substrate and culture media. Before the final collection of the HCF, control cell monolayers are examined microscopically throughout the harvest period.]

Post-infection, the virus propagators are refed and the spent medium is drained and discarded; the virus harvest is collected. The HVFs are sampled for virus potency and sterility and shell frozen.

Controls of materials and critical steps / process validation

The seed testing is consistent with the Ph. Eur., Section 2.6.16 and monograph for Mumps Vaccine (Live), with the exception of the virus identification test. Identity testing is instead performed post- clarification on the vaccine bulk, where antibody neutralization can be performed on a clarified bulk virus solution.

Definition of CPPs and process validation were performed in a similar manner as for measles.

Characterisation and specifications

To assess the population diversity of the stock seed and bulk product, the JL-strain specific nucleotide sequences were determined and results provided in the dossier.

Process-related impurities arising from the mumps bulk manufacturing processes may be classified as cell substrate-derived or cell culture-derived. Since the mumps process uses cell growth medium containing fetal bovine serum (FBS), mumps bulk lots were tested for BSA and the results for all of these lots were within the specification. Mumps vaccine is an unpurified product whose potency is measured through a biological assay for the active substance rather than through evaluation of integrity of physical form. Degradation products are neither identified nor quantified.

The testing (and method validation) of the mumps bulk is essentially the same as for the measles bulk. Batch analysis results have been provided for HVF/HCF lots and dispensed bulk lots; all results met specifications.

The reference standard used in potency testing is a monovalent mumps vaccine lot manufactured using currently approved processes. The applicant committed to characterize the performance of the mumps potency assay with international reference standards.

ACTIVE SUBSTANCE - RUBELLA

Seed lot system

The Wistar RA 27/3 strain of rubella virus was isolated in 1964 by Dr. Stanley Plotkin, Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania, U.S., from a kidney explant obtained from a surgically aborted foetus. It was directly inoculated into WI-38 cells, and then attenuated.

Human diploid fibroblast cells (MRC-5) as cell substrate

MRC-5 cells, a human, embryonic, lung, fibroblast cell line (diploid, male) originally isolated by J.P. Jacobs at the National Institute for Medical Research (London, England) and deposited at approximately population doubling level (PDL) 7 at the National Institute for Biological Standards and Controls (NIBSC).

Characterisation and specifications

Rubella virus Stock Seed Lots showed complete agreement in the nucleotide sequence alignment.

Process-related impurities arising from the rubella vaccine bulk manufacturing processes are classified as cell-substrate or cell-culture derived. Cell-substrate-derived impurities may include proteins derived from the host cell line; cell-culture-derived impurities may include antibiotics (e.g., neomycin), serum, or other media components.

Rubella vaccine bulk is an unpurified product whose potency is measured through a biological assay for the active substance rather than through evaluation of integrity of physical form. Degradation products have been neither identified nor quantified.

Drug substance release tests are performed at the specified stages of vaccine bulk processing in order to confirm absence of extraneous agents, to verify potency and identity, and to provide a measure of quality and process consistency. For qualitative assays, the specifications are based on historical data. Assays involved in control of drug substance are performed according to approved control procedures that describe the main steps in a procedure.

The validation was performed using the assay procedure that was in place at the time the assays were validated. The parameters that were evaluated as part of the method validation for the assays were provided for each analytical procedure.

Batch analysis results have been provided for three HVFs, pooled bulk lots and ProQuad filled container lots. Consistency of production was demonstrated and all lots met the specifications.

The reference standard used in potency testing is a monovalent rubella vaccine lot manufactured using the currently approved process. The applicant committed to characterize the performance of the rubella potency assay with international reference standards.

ACTIVE SUBSTANCE - VARICELLA

Seed lot system

The Oka strain of the varicella –zoster virus (VZV) was isolated from fluid taken from the vesicles of a 3-year-old boy with a case of chicken pox. The virus was isolated in primary human, embryonic lung cells (HEL) and was passaged 11 times. The strain was further passaged 12 times in guinea pig embryo fibroblasts (GPE) to attenuate the strain and once in human diploid cells (WI-38) to passage 24.

Human diploid fibroblast cells (MRC-5) as cell substrate

MRC-5 cells, a human, embryonic, lung, fibroblast cell line (diploid, male) originally isolated by J.P. Jacobs at the National Institute for Medical Research (London, England) and deposited at approximately population doubling level (PDL) 7 at the National Institute for Biological Standards and Controls (NIBSC).

Manufacture of varicella harvested virus fluids (HVFs)

A vial from the MWCB is thawed and planted. Cells are trypsinized and finally planted for infection. A batch of HVF represents mechanically harvested, varicella-infected MRC-5 cells.

Based on appropriate criteria, the concentration of the working seed is adjusted. Each production roller bottle is planted with working seed cell suspension and incubated. The spent medium is removed and discarded, and each cell culture is rinsed. Stabilizer is added; the suspension is removed and stored with appropriate conditions.

Manufacture of dispensed bulk

Varicella dispensed bulk is a blend of HVF lots. The cells in the HVF suspension are disrupted and clarified. This volume is dispensed prior to freezing. The dispensed final bulk containers (dispensed bulk) comprise a batch of the active substance and are stored.

Control cell Cultures and Harvest Control Fluids (HCFs)

Final harvested control fluids are tested for sterility, mycoplasmas, and tissue culture safety, while cells are tested for hemadsorption.

Controls of materials and critical steps / process validation

The MRC-5 MCB and WCB are tested to ensure freedom from extraneous agents and to ensure that the cells behave normally through production use PDL. Release testing is described at appropriate process steps and will be performed in compliance with Ph. Eur 5.2.3.

Release testing of the varicella master seed and stock seeds is performed in compliance with Ph. Eur. The applicant committed to consult the EMEA to discuss the need for monkey neurovirulence testing on any new varicella master seed if MNV is still required in the Ph. Eur.

Within each manufacturing process step, goals, CPPs, and CQAs were determined, along with appropriate specifications and acceptance criteria.

Characterisation and specifications

The complete sequences of the Oka/Merck strain and the wild-type Oka parent have been determined. Process-related impurities arising from the rubella vaccine bulk manufacturing processes are classified as cell-substrate or cell-culture derived. Cell-substrate-derived impurities may include proteins derived from the host cell line; cell-culture-derived impurities may include antibiotics (e.g., neomycin), serum, or other media components. Varicella process uses cell growth medium containing fetal bovine serum. Serum protein clearance is provided by rinsing the cell layers to remove as much serum as possible prior to virus harvest. Each varicella final bulk is tested for BSA.

Assays are performed at several stages of processing of vaccine bulks in order to confirm absence of extraneous agents, to verify potency and identity, and to provide a measure of quality and process consistency. Assays involved in release testing of drug substance are performed according to approved control procedures that describe the main steps in a procedure. Most assays performed on varicella vaccine bulks and bulk intermediates are qualitative methods for which the experimental outcome is only: growth or no growth, absence or presence etc... In many of these cases, the assay specifications are compendial. For quantitative assays, the acceptance criterion is based on historical data.

The validation was performed using the assay procedure that was in place at the time the assays were validated. The parameters that were evaluated as part of the method validation for the assays were provided for each analytical procedure.

Batch analysis results have been provided for three HVFs, pooled bulk lots and ProQuad filled container lots. Consistency of production was demonstrated and all lots met the specifications.

FINISHED PRODUCT

We refer to ProQuad for the product specification, because the monographs and the guidelines have to be the same for Priorix tetra

Product Specification

The testing scheme of the finished product represents a combination of the regimens used for the testing of measles, mumps, rubella, and varicella virus-containing vaccines, all of which are in currently licensed products.

Tests are performed on the drug product to ensure safety, sterility, to confirm the identity and quantify the potency of the product, and to provide a measure of process consistency. Assays employed in control of the finished product lots are performed according to approved CPs that describe the main steps in a procedure.

Each dose of the vaccine contains at the end of its shelf-life a minimum of 3.00 log TCID₅₀ measles virus, 4.30 log TCID₅₀ mumps virus, 3.00 log TCID₅₀ rubella virus, and 3.99 log plaque forming unit (PFU) varicella virus. The release specifications have been selected to ensure that, at expiry, each dose will contain the aforementioned minimum potency for each virus when the vaccine is reconstituted and stored at room temperature for 30 minutes.

Several assays performed on the finished product are qualitative methods for which there are only two experimental outcomes (growth or no growth, absence or presence, etc.). In many cases, the assay specifications are compendial.

The potency specifications for filled container have been derived from several sources. Each virus release potency is described in the dossier.

The parameters that were evaluated as part of the method validation for the assays are listed for each analytical procedure. When applicable, the assay parameters addressed were specificity, inter-assay precision, limit of detection, limit of quantitation, linearity, range, ruggedness, and robustness.

Batch analysis was performed on three process validation lots within the range of a commercial lot size. All results met the pre-defined specifications.

Because ProQuad is a live virus vaccine composed of measles, mumps, rubella and varicella bulks prepared from cell culture fluids, it is not a highly purified product. To provide a marker for removal of fetal bovine serum used during the cell culture process, a quantitative test for residual BSA is conducted on the virus bulks. This BSA content is used to calculate the amount of BSA present in the filled container based on the dilution of each bulk during filling. A specification exists for BSA content in filled container (≤ 500 ng BSA per single human dose) as per the Ph. Eur. monograph 0648 even though filled container material is not directly tested.

The applicant committed to characterize the performance of his potency assay with international reference standards and to establish a 'gold standard' with a link to the clinic for calibration of future standards used in ProQuad potency testing.

Viral safety and TSE

Adventitious Agents

The testing program for adventitious agents is described in detail in the chapters on the Measles, Mumps, Rubella, and Varicella active substances. All raw materials used in vaccine manufacturing are tested for adventitious agents prior to release and use in manufacturing.

Validated processing steps that add additional levels of confidence for the absence of adventitious agents are filter sterilization and ultraviolet (UV)- or gamma -irradiation.

TSE

The manufacturing process for ProQuad™ was evaluated for the theoretical risk of transmission of infectivity associated with BSE prions, with the conclusion that the risk of BSE transmission in ProQuad is exceedingly remote. The rationale and the calculation for the theoretical risk of transmission of infectivity associated with BSE prions were provided.

Biological reagents used in the manufacture of the vaccine or intermediates include iron-enriched bovine calf serum (BCS), fetal bovine serum (FBS), porcine pancreatic trypsin, porcine-derived hydrolyzed gelatine, choline chloride, bovine or porcine tallow-derived polysorbate 80, fish or sheep wool-derived cholesterol, amino acids, and human serum albumin (HSA). Certificates of Suitability (CoS), which are granted by the European Directorate for the Quality of Medicines (EDQM), and the measures applied (e.g. regular audits of vendor facilities, testing to ensure that the appropriate quality standards are met, etc.) ensure that the ruminant-derived raw materials currently used in manufacturing are free of transmissible spongiform encephalopathy (TSE) or bovine spongiform encephalopathy (BSE) contamination.

Estratti delle risposte dell'EMA alle nostre richieste per informazioni relative alla qualità dei vaccini

EMA request reference ASK-51104 - 21 mar 2019

(...) Per quanto riguarda la sua richiesta di avere dettagli sul controllo di qualità dei vaccini, può trovare linee guida relative allo sviluppo e alla produzione di vaccini (farmaci biologici) sul sito web dell'Agenzia:

<https://www.ema.europa.eu/en/human-regulatory/research-development/scientific-guidelines/biologicals/biologicals-finished-product>
<https://www.ema.europa.eu/en/human-regulatory/research-development/scientific-guidelines/multidisciplinary/multidisciplinary-vaccines>
<https://www.ema.europa.eu/en/human-regulatory/research-development/scientific-guidelines/ich/ich-quality>

I vaccini sono tenuti a seguire le monografie pertinenti della Farmacopea europea.

Questi sono disponibili dall'EDQM: <https://www.edqm.eu/>

I dettagli del processo di produzione e dei controlli, inclusi i test analitici e i risultati, fanno parte del dossier di autorizzazione all'immissione in commercio e sono conformi alle linee guida e agli standard pertinenti al momento dell'autorizzazione. Il dossier fa parte del documento tecnico comune (CTD) e i dettagli di fabbricazione sono inclusi nel Modulo 3. Va notato che **la maggior parte delle informazioni del Modulo 3 è considerata commercialmente riservata e l'agenzia non è in grado di rilasciare questo su richiesta di una terza parte.**

Per quanto riguarda i dati di rilascio del lotto (certificati di analisi citati nella domanda), vale a dire test analitici in base alle specifiche del prodotto, si prega di notare il processo per questo nell'UE.

Tutti i lotti di vaccini autorizzati dall'UE sono rilasciati dal titolare dell'autorizzazione all'immissione in commercio con saggi approvati che dimostrano di determinare adeguatamente l'efficacia (ossia l'attività biologica) di tutti i principi attivi inclusi, nonché altri parametri considerati rilevanti e critici per tale prodotto (ad esempio sterilità, endotossina, pH, contenuto proteico residuo, ecc.), parametri che possono variare in base alle caratteristiche specifiche di ciascun prodotto. Inoltre, tutti i vaccini approvati tramite EMA e attraverso una procedura di mutuo riconoscimento, e molti di quelli approvati a livello nazionale, richiedono anche il rilascio dei lotti da parte di uno dei laboratori della rete OMCL (Official Medicines Control Laboratory) prima di consentire alla società di commercializzare il lotto in un territorio specifico.

Per maggiori informazioni:

<https://www.edqm.eu/en/batch-release-human-biologicals-vaccines-blood-and-plasma-derivatives>

Questi laboratori sono coordinati dalla Direzione Europea per la Qualità dei Medicinali (EDQM), che pubblica anche la Farmacopea Europea. Le analisi eseguite dai laboratori OMCL sono specifiche per i prodotti vaccinali e la procedura di rilascio dei lotti è stabilita in linee guida specifiche, elencate sul sito Web di EDQM (<https://www.edqm.eu/en/human-ocabr-guidelines>). In ogni linea guida, sono indicate le specifiche monografie della Ph. Eur. monografie rilevanti per ciascun tipo di vaccino.

Per Infanrix Hexa, Hexyon, Priorix Tetra e Gardasil 9 il seguente specifico Ph. Eur. Si applicano le monografie: n. 2067 (Infanrix Hexa ed Hexyon), n. 1057 e 0648 (Priorix Tetra), n. 2441 (Gardasil 9).

La Ph. Eur. è disponibile sul sito Web di EDQM al seguente indirizzo:

<https://www.edqm.eu/en/european-pharmacopoeia-ph-eur-9th-edition>.

I test che devono essere eseguiti dai produttori per il rilascio di ciascun lotto e le sue principali fasi di produzione, vengono anche sottoposti all'OMCL per la revisione. **Si noti che il sistema europeo di rilascio in lotti di medicinali biologici funziona secondo una base di riconoscimento reciproco, ossia quando un lotto di vaccino viene testato da una delle OMCL, il certificato rilasciato, di conformità o non conformità, è riconosciuto e accettato in tutti gli altri paesi dell'UE in cui il prodotto è commercializzato.**

In linea con quanto descritto in precedenza, **l'EMA non effettua test analitici sui medicinali**, come parte del rilascio ufficiale di medicinali biologici, poiché questa è una responsabilità della rete OMCL. Questi dati possono essere resi disponibili all'Agenzia se un problema specifico richiede la loro valutazione.

Infine, per quanto riguarda l'organizzazione di un incontro con l'Agenzia, questo può essere considerato a tempo debito. Come accennato in precedenza, avremmo bisogno di vedere una chiara presentazione del problema nella documentazione di fondo, che potrebbe essere condivisa con esperti dell'UE per capire se ci sono motivi da prendere in considerazione, cioè se ci sono problemi di qualità associati ai vaccini che ha citato e inoltre se vi è motivo di ritenere che la sicurezza dei vaccini sia compromessa.

Apprezziamo le preoccupazioni che sorgono riguardo alla vaccinazione e ai medicinali in generale e l'Agenzia sarà lieta di ricevere e valutare tali preoccupazioni quando riceveremo la sua ulteriore comunicazione.

Cordiali saluti,
Ufficio di qualità

Agenzia europea per i medicinali
Indirizzo ufficiale: Domenico Scarlattilaan 6, 1083 HS Amsterdam, Paesi Bassi

EMA request reference ASK-51112 - 19 mar 2019

ASK-51112 19 mar 2019

(...) Al fine di soddisfare ulteriormente la richiesta della sua email di seguito, ho eseguito un controllo dettagliato dei documenti che EMA detiene come previsto dal titolare dell'autorizzazione all'immissione in commercio.

Ho controllato l'ultima sequenza dei documenti inviati. Quest'ultima sequenza copre le procedure iniziali e anche quelle successive all'autorizzazione.

Più in particolare, il controllo è stato effettuato sul Modulo 3 con particolare attenzione ai sottomoduli 3.2.S.2.3 (materie prime e terreni di coltura), 3.S.2.4, 3.2.P.4.1 (specifiche), 3.2.A.3 Eccipienti.

Oltre a questo controllo, si prega di notare che non possediamo, monografie di Ph. Eur o qualsiasi altra monografia (ad esempio USP: United States Pharmacopeia, NF: National Formulary (Stati Uniti)).

È possibile trovare informazioni pertinenti su monografie / specifiche degli ingredienti / eccipienti ecc. nell'EPAR del prodotto in questione.

Vedi ad esempio sotto una raccolta che potresti trovare utile. Questa raccolta di riferimenti EPAR non è esaustiva, quindi si prega di approfondire nel dettaglio il link EPAR fornito:

Products	Information on Ph. Eu. Monographs, Monographs and the specifications used
Infanrix hexa	<p>See EPAR Page 4: Hib component</p> <p>The active ingredient used for the preparation of the final bulk is the adsorbed PRP-T conjugate bulk, which is manufactured in compliance with the Ph. Eur. monograph on Haemophilus influenzae type b conjugate vaccines and with WHO requirements for the same vaccine.</p> <p>https://www.ema.europa.eu/en/documents/scientific-discussion/infanrix-hexa-epar-scientific-discussion_en.pdf</p>
Gardasil 9	<p>See EPAR page 16, Complete product composition. See EPAR page 18, product specification.</p> <p>https://www.ema.europa.eu/en/documents/assessment-report/gardasil-9-epar-public-assessment-report_en.pdf</p>

Hexyon

See EPAR Page 17

Specification

The control of the drug substance complies with WHO TRS 786 and Ph. Eur. monograph 1056.

See EPAR Page 21, product specification

See EPAR Page 11:

In process controls (IPCs) for the intermediates of the drug substance include tests with specified acceptance criteria and tests to monitor the process. All IPCs applied are in compliance with the bulk purified toxoid part of Ph. Eur. monograph 0443 "Diphtheria vaccine (adsorbed)", and with WHO TRS No. 800 Annex 2 "Requirements for diphtheria, tetanus, pertussis and combined vaccines (adsorbed)".

See EPAR Page 12, Specification

The tests and specifications for the control of the PDT drug substance are in compliance with the bulk purified toxoid part of Ph. Eur. monograph 0443 "Diphtheria vaccine (adsorbed)", and with WHO TRS No. 800 Annex 2 "Requirements for diphtheria, tetanus, pertussis and combined vaccines (adsorbed)".

See EPAR Page 13, Specification

The specifications for the PTT drug substance are in compliance with the Ph. Eur. monograph 0452 and with WHO TRS 800. Batch Analyses performed on 3 clinical batches as well as on 3 current production batches met acceptance criteria and showed consistency and uniformity

https://www.ema.europa.eu/en/documents/assessment-report/hexyon-epar-public-assessment-report_en.pdf

Dr Valentina Stamouli
Access to Documents Manager
Office of the Deputy Executive Director

EMA request reference ASK-39927 - 4 Apr 2018

Dear Ms Bolgan

Thank you for your query on human vaccines which is addressed below.

• **i limiti di sicurezza sui contaminanti (chimici, biologici, elementari) e le linee guida di riferimento**

Per quanto riguarda i residui (impurità legate al processo) nel prodotto finale, la procedura di fabbricazione influenzerà la natura, la gamma e la quantità del potenziale residuo nel prodotto finale e i processi di purificazione devono essere in atto per ridurli o rimuoverli

(Vedi produzione e controllo di qualità dei medicinali derivati dalla tecnologia del DNA ricombinante

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003433.pdf).

I residui risultanti dal processo di fabbricazione come proteine delle cellule ospiti, acidi nucleici, componenti dei media, virus avventizi e reagenti utilizzati nella fabbricazione sono accuratamente valutati e ridotti a livelli accettabili. Per ulteriori informazioni sulla purificazione fare riferimento alle Linee guida sulla convalida del processo per la fabbricazione di sostanze attive e dati derivati dalle biotecnologie da fornire nella presentazione al regolatorio (i principi si applicano ai vaccini in base alla natura specifica del prodotto

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/04/WC500205447.pdf.

Limiti specifici per i residui possono dipendere dal processo, tuttavia esistono requisiti per le avvertenze sull'etichetta del prodotto in circostanze specifiche per una serie di sostanze (vedere Eccipienti nell'etichetta e nel foglio illustrativo dei medicinali per uso umano

https://ec.europa.eu/health/sites/health/files/files/eudralex/vol-2/c/guidelines_excipients_july_2013_rev_1.pdf

e il suo allegato

http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_001646.jsp).

Inoltre, c'è una guida per la descrizione nelle informazioni sul prodotto di una serie di sostanze di uso comune lasciate dal processo di fabbricazione (consultare le Linee guida sugli aspetti di qualità incluse nelle informazioni sul prodotto per i vaccini per uso umano. Questo collegamento è la bozza revisionata aperta per consultazione

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2018/02/WC500242956.pdf.

Ci sono alcuni componenti citati nelle monografie dei vaccini della Farmacopea Europea (Ph.Eur.)

(Vedi <https://www.edqm.eu/en/european-pharmacopoeia-ph-eur-9th-edition>), un compendio che elenca le sostanze attive e gli ingredienti utilizzati per preparare prodotti farmaceutici in Europa e che viene utilizzato come riferimento ufficiale per il controllo di qualità dei medicinali in Europa. (...)

Si noti inoltre che limiti specifici per il DNA residuo delle cellule ospiti, le proteine delle cellule ospiti (ad esempio, l'ovalbumina o l'albumina sierica bovina residua [BS]) o altre sostanze possono essere specificate nelle monografie specifiche del vaccino della Ph.Eur. Per qualsiasi informazione relativa a queste monografie della Ph. Eur., contattare la direzione europea per la qualità dei medicinali e della sanità (EDQM).

Vedi <https://www.edqm.eu/en/EDQM-contact-685.html>. (...)

• **i limiti di tolleranza per le cellule fetali umane nei vaccini MMRV e le cellule embrionali sui vaccini antinfluenzali e le linee guida di riferimento**

A seguito di un parere scientifico emesso dal Comitato per i medicinali per uso umano (CHMP) dell'Agenzia dei medicinali (EMA), la Commissione europea ha concesso due autorizzazioni all'immissione in commercio centralizzate di vaccini per MMR e / o varicella. Questi sono M-M-RVAXPRO (vaccino contro morbillo, parotite e rosolia (vivo)) e ProQuad (vaccino contro morbillo, parotite, rosolia e varicella (vivo)). Per ulteriori informazioni sull'approvazione di questi vaccini nell'UE, consultare i link di riferimento:

http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000604/human_med_000907.jsp&mid=WC0b01ac058001d124

http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000622/human_med_000997.jsp&mid=WC0b01ac058001d124

Per entrambi questi vaccini, il virus della rosolia è stato prodotto nella linea cellulare diploide umana (WI-38- vedi Ph.Eur. Capitolo 5.2.3. Substrati cellulari per la produzione di vaccini per uso umano per informazioni su questo tipo di linea cellulare) e i componenti della parotite e del morbillo sulle cellule embrionali di pollo. Per Proquad, il virus varicella (varicella) è stato prodotto nella linea cellulare diploide umana (MRC-5). Ad altri vaccini MMR e / o varicella potrebbe essere stata concessa l'autorizzazione all'immissione in commercio negli Stati membri dell'UE attraverso le procedure nazionali di autorizzazione all'immissione in commercio anziché la procedura centralizzata di autorizzazione all'immissione in commercio. Se si desidera ottenere ulteriori informazioni sui vaccini autorizzati a livello nazionale, è possibile contattare l'autorità nazionale competente degli Stati membri dell'UE di proprio interesse. I dettagli di contatto delle autorità nazionali competenti sono disponibili tramite il link di riferimento

(http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/general/general_content_000155.jsp&murl=menus/partners_and_networks/partners_and_networks.jsp&mid=WC0b01ac0580036d63).

(...)

L'uso di linee cellulari diploidi umane come substrato per la produzione di vaccini virali è ben consolidato dal punto di vista della qualità, della sicurezza e dell'efficacia. A parte le rispettive monografie della Ph.Eur., che potrebbero fissare limiti specifici per i residui, la valutazione delle impurità e dei contaminanti virali endogeni viene presa in considerazione durante la revisione del processo di fabbricazione di questi prodotti in base ai livelli utilizzati nel prodotto per gli studi clinici e in base alla valutazione del rapporto rischio / beneficio dell'uso del prodotto. Per ulteriori informazioni si consiglia di consultare i documenti di riferimento (impurità del DNA e delle proteine delle cellule ospiti, test di routine verso studi di validazione

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003322.pdf

Raccomandazioni per la valutazione delle colture cellulari animali come substrati per la fabbricazione di medicinali biologici e per la caratterizzazione delle banche cellulari, Organizzazione mondiale della sanità, 2010.

http://www.who.int/biologicals/Cell_Substrates_clean_version_18_April.pdf

• **il limite massimo consentito di mutazioni genetiche sugli antigeni del vaccino e se è possibile un collegamento di riferimento dove trovare la sequenza genetica degli antigeni del vaccino attualmente sul mercato**

I vaccini per uso umano possono contenere: microrganismi interi (batteri, virus o parassiti), inattivati con mezzi chimici o fisici che mantengono adeguate proprietà immunogene; microrganismi vivi interi che sono naturalmente avirulenti o che sono stati trattati per attenuare la loro virulenza pur mantenendo adeguate proprietà immunogene; antigeni estratti dai microrganismi o secreti dai microrganismi o prodotti mediante ingegneria genetica o sintesi chimica. Gli antigeni possono essere usati nel loro stato nativo o possono essere detossificati o altrimenti modificati con mezzi chimici o fisici e possono essere aggregati, polimerizzati o coniugati a un veicolo per aumentare l'immunogenicità (Vedi monografia sui vaccini per uso umano (Ph.Eur. 07 / 2017 0153)). Data la complessità, quindi, dei vaccini per uso umano, i produttori sono tenuti a giustificare la struttura del principio attivo di un vaccino specifico durante la domanda di autorizzazione all'immissione in commercio e a presentare dati di

caratterizzazione adeguati a supporto di ciò. Il principio attivo deve essere dimostrato sicuro ed efficace negli studi clinici prima dell'approvazione della domanda di autorizzazione all'immissione in commercio. Consultare le monografie specifiche del prodotto di Ph.Eur., che possono fornire ulteriori informazioni sulla caratterizzazione richiesta per tipi specifici di vaccino. L'Agenzia non pubblica alcuna informazione sulle sequenze genetiche di un antigene vaccino poiché è considerata commercialmente confidenziale. Vari motori di ricerca nella letteratura medico / scientifica possono essere utilizzati per verificare se la società / altri investigatori hanno sequenziato i componenti antigenici di specifici vaccini.

Ci auguriamo che le informazioni sopra riportate siano utili.

Cordiali saluti

L'ufficio qualità.

EMA/133566/2018 - 16 March 2018

Gentile signor Catalano

1.2. residui

Per quanto riguarda i residui (impurità legate al processo) nel prodotto finale, la procedura di fabbricazione influenzerà la natura, la gamma e la quantità del potenziale residuo nel prodotto finale e i processi di purificazione devono essere in atto per rimuoverli.¹

In generale, durante la valutazione di un vaccino, le procedure di purificazione del processo di fabbricazione per rimuovere i residui risultanti dal processo di fabbricazione (ad esempio varianti indesiderate, proteine delle cellule ospiti, acidi nucleici, componenti dei media, virus e reagenti utilizzati nella modifica della proteina) a livelli accettabili è valutato attentamente. Per ulteriori informazioni, fare riferimento alle Linee guida sulla convalida del processo per la fabbricazione di sostanze attive e dati derivati dalle biotecnologie da fornire nella presentazione normativa (l'ambito di applicazione esclude i vaccini, ma i principi della guida possono applicarsi a seconda della natura specifica del prodotto).²

Si noti inoltre che limiti specifici per il DNA residuo delle cellule ospiti, le proteine delle cellule ospiti (ad esempio, l'ovalbumina o l'albumina sierica bovina residua [BSA]) o altre sostanze possono essere specificati nelle monografie specifiche del vaccino del Ph.Eur. Di seguito sono riportati alcuni esempi e questi non sono esaustivi. Inoltre, si noti che questi limiti potrebbero non essere necessariamente applicabili ai vaccini combinati, quindi le monografie specifiche di vaccini combinati del Ph.Eur. dovrebbero essere consultate. Per qualsiasi informazione in merito, si prega di contattare la direzione europea per la qualità dei medicinali e della sanità (EDQM) che pubblica il Ph.Eur.³

1.3. DNA delle cellule ospiti e proteine delle cellule ospiti (HCP)

Non esistono limiti europei obbligatori uniformi applicabili a tutti i medicinali.⁴ Tuttavia, esistono monografie specifiche di vaccini nella Farmacopea Europea che stabiliscono limiti per alcuni vaccini, ad es. per il vaccino inattivato e adsorbito per l'epatite A (non combinato con altri vaccini), se viene utilizzata una linea cellulare continua per la propagazione del virus, il contenuto del DNA residuo della cellula ospite non deve essere superiore a 100 picogrammi nell'equivalente di una singola dose umana. Un limite simile è dato per il vaccino contro il rotavirus (vivo, orale). Per il vaccino contro l'epatite B (rDNA) (non combinato con altri vaccini) per il DNA derivato da cellule ospiti e da vettori, se per la produzione vengono utilizzate cellule di mammifero, non più di 10 picogrammi di DNA nella quantità di antigene purificato equivalente a una singola dose umana di vaccino è ammessa. Un limite simile è anche dato per il vaccino contro il papillomavirus umano (rDNA), il vaccino contro il vaiolo (vivo), il vaccino contro la rabbia preparato in coltura cellulare e i vaccini contro l'influenza propagata su cellule.

Inoltre, al fine di mitigare i potenziali effetti avversi (ad es. Immunogenicità), il contenuto di HCP deve essere sempre ridotto al livello più basso possibile. L'autorizzazione dell'HCP durante il processo di purificazione deve essere valutata e il contenuto dell'HCP determinato mediante un test HCP che è stato valutato e validato per un determinato prodotto. Dovrebbe anche consultare monografie specifiche di interesse della Farmacopea europea in quanto potrebbero specificare dei limiti. Ad esempio, per il vaccino antinfluenzale (vivo, nasale) e altri vaccini antinfluenzali propagati sull'uovo e vaccini contro la parotite-morbillo-rosolia, viene specificato un limite per l'ovalbumina non superiore a 1 microgrammo per dose umana. Per il vaccino contro la febbre gialla (vivo) vengono impostati al massimo 5 microgrammi di ovalbumina per dose umana.

¹ Production And Quality Control Of Medicinal Products Derived By Recombinant DNA Technology

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003433.pdf

² Guideline on process validation for the manufacture of biotechnology-derived active substances and data to be provided in the regulatory submission- scope excludes vaccines, but principles of the guidance may apply depending on the specific nature of the product.

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2016/04/WC500205447.pdf

³ <https://www.edqm.eu/en/EDQM-contact-685.html>

⁴ DNA and host cell protein impurities, routine testing versus validation studies

http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003322.pdf

EMA request reference ASK-43967 3 August 2018

Gentile Sig. Donolato,

La ringraziamo per la sua mail del 16 luglio 2018, nella quale esprime i suoi dubbi sulla qualità di alcuni vaccini autorizzati nell'Unione Europea. In risposta ai suoi timori che alcuni vaccini nell'UE siano contaminati con DNA cellulare o virale, la informiamo che, come nel caso di tutti i farmaci biologici, i vaccini possono contenere quantità residue di DNA. Esse derivano dai processi di produzione e non sono considerate sostanze contaminanti, ma residui. Tali residui che si formano durante il processo di produzione vengono attentamente valutati dalle autorità regolatorie durante le procedure di valutazione per l'autorizzazione all'immissione sul mercato e ridotti a livelli accettabili.

L'Agenzia Europea dei Medicinali (EMA) prende molto sul serio il suo ruolo di proteggere la salute pubblica. Come nel caso di qualsiasi medicinale autorizzato a seguito di una analisi scientifica da parte dell'EMA, la qualità, la sicurezza e l'efficacia dei vaccini sono attentamente valutate, in conformità con i requisiti della legislazione, prima che venga loro concessa l'autorizzazione all'immissione in commercio.

Inoltre, l'EMA monitora tutti i medicinali presenti sul mercato e quindi la qualità, la sicurezza e l'efficacia di tutti i vaccini sono costantemente monitorate anche dopo la loro autorizzazione.

Per quanto riguarda i vaccini Priorix Tetra, Vivotif e Measles live B.P., questi sono stati autorizzati nei vari paesi dell'UE tramite procedure di autorizzazione nazionali e non tramite l'EMA. Se desidera ottenere maggiori informazioni su vaccini autorizzati a livello nazionale, può contattare l'autorità nazionale competente negli Stati Membri dell'UE di suo interesse. I contatti delle autorità nazionali competenti sono disponibili al seguente link:

http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/general/general_content_000155.jsp&murl=menus/partners_and_networks/partners_and_networks.jsp&mid=WC0b01ac0580036d63

Le segnaliamo che la Farmacopea Europea (Eur. Ph.) descrive gli standard qualitativi ufficiali a cui i produttori devono attenersi per le materie prime utilizzate nella produzione di medicinali. In particolare, il capitolo 5.2.3 sui substrati cellulari per la produzione di vaccini per uso umano contiene informazioni sui tipi di linee cellulari che possono essere utilizzati e sui requisiti riguardo ai test e alle specifiche.

In base al capitolo 5.2.3 deve essere stabilito un limite accettabile per il DNA residuo della cellula ospite (che è specifico del prodotto e dipende da diversi fattori tra cui la natura del substrato cellulare/le caratteristiche del processo di produzione/il tipo di prodotto, ecc.) solo quando il processo di produzione utilizza determinate linee cellulari con la capacità di moltiplicarsi indefinitamente in vitro (cioè linee cellulari continue). Come indicato nello stesso capitolo, "Per i vaccini prodotti in linee cellulari continue, sia tumorigeniche sia non tumorigeniche, occorre eseguire la valutazione del rischio e la mitigazione del rischio per valutare l'idoneità del substrato cellulare, per definire i criteri accettabili per il DNA residuo di cellule ospite nel prodotto finale e per valutare la consistenza delle proteine delle cellule ospite."

Sulla base delle informazioni pubblicate, Priorix-Tetra contiene ceppi virali prodotti separatamente in cellule di embrioni di pollo (parotite e morbillo) o in cellule diploidi umane MRC-5 (rosolia e varicella). Le linee cellulari utilizzate per Priorix Tetra includono linee di cellule diploidi umane che non possono dividersi continuamente. Si noti che, secondo la Farmacopea Europea, le linee di cellule diploidi MRC-5 non sono tumorigeniche, come dimostrato da decenni di uso e controllo, e pertanto non si applica un limite massimo per il DNA di cellule MRC-5.

Le consigliamo di consultare anche il documento dell'OMS "Raccomandazioni per la valutazione delle colture di cellule animali come substrati per la produzione di farmaci biologici e per la caratterizzazione di banche cellulari (2013)" che fornisce ampi chiarimenti sui rischi associati ai vari tipi di cellule utilizzate nella produzione di vaccini e conferma ancora una volta che "Le linee di cellule diploidi sono state usate con successo per molti anni per la produzione di vaccini virali e il DNA cellulare residuo derivante da queste cellule non è stato (e non è) considerato di porre alcun rischio significativo." Per maggiori informazioni al riguardo, le consigliamo di consultare il seguente link:

http://www.who.int/biologicals/vaccines/TRS_978_Annex_3.pdf?ua=1

Per quanto riguarda la possibilità che le varianti genetiche influenzino la sicurezza o l'efficacia, le segnaliamo che i cambiamenti nella sequenza genomica di un virus non hanno necessariamente un impatto su quei determinanti antigenici necessari per stimolare la risposta immunitaria desiderata nell'uomo. Analogamente, il mantenimento dell'attenuazione dei virus contenuti in questi vaccini, vengono specificamente controllato durante la valutazione della domanda di autorizzazione all'immissione in commercio.

Tutti i vaccini autorizzati nell'UE sono testati prima della distribuzione dei lotti dal titolare dell'autorizzazione all'immissione in commercio, utilizzando test approvati che misurano adeguatamente la potenza (cioè l'attività biologica) di tutti i principi attivi inclusi. Inoltre, tutti i vaccini approvati tramite l'EMA e molti di quelli approvati a livello nazionale richiedono anche dei test prima della distribuzione dei lotti da parte di un

laboratorio ufficiale di controllo dei farmaci per consentire all'azienda di commercializzare i lotti in un territorio specifico. Per ulteriori informazioni, consultare il seguente link:

<https://www.edqm.eu/en/batch-release-human-biologicals-vaccines-blood-and-plasma-derivatives>

Per quanto riguarda Infanrix hexa, che è stato autorizzato tramite l'EMA, questo è un vaccino che contiene tre poliovirus inattivati (tipi 1,2 e 3), oltre ad altri antigeni. Il test di rilascio di questo vaccino è fatto in linea con i requisiti dell'OMS e della Ph. Eur, i quali prevedono l'analisi dell'attività biologica della poliomielite. Ciò viene fatto misurando l'antigene D (l'antigene D del poliovirus provoca anticorpi neutralizzanti). La poliomielite è un virus RNA e come indicato nel riassunto della valutazione scientifica

(http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000296/WC500032501.pdf)

il DNA delle cellule ospite viene eliminato durante il processo di purificazione (in questo caso le cellule ospite sono cellule Vero, cioè una linea cellulare continua isolata dalle cellule epiteliali della scimmia). Quindi, il DNA cellulare non contribuisce all'effetto di questo vaccino, ma tutti gli antigeni necessari per la sua efficacia sono presenti.

Ci auguriamo che queste informazioni le siano utili.

Cordiali saluti,

Federica Castellani

Stakeholders and Communication Division

Dossier EMA - NGS

Discussione dei risultati presentati

Discussione dei risultati presentati

Lo studio commissionato dal Corvelva si è articolato su due tipi di analisi:

1. Test di presenza di acidi nucleici (DNA/RNA) di origine umana e animale e da microrganismi (virus, batteri) utilizzando la metodica Next Generation Sequencing, che ha permesso di quantificare in maniera altamente specifica e accurata la sequenza del materiale genetico contenuto nei vaccini esaminati.
2. Verifica di corrispondenza delle sequenze genomiche dei batteri e virus vivi attenuati o inattivati presenti nei vaccini (presenza di varianti genetiche)

Descrizione del metodo utilizzato per l'analisi

Il Next Generation Sequencing, ³ noto anche come deep sequencing o sequenziamento massivo parallelo è una tecnologia innovativa e altamente versatile che permette il sequenziamento in parallelo di milioni di frammenti di DNA. L'analisi bioinformatica a valle consente poi la differenziazione tra l'origine dei frammenti di sequenza, ad esempio umana, specie batteriche o un particolare virus. Questo significa che campioni biologici misti possono essere agevolmente risolti con questa tecnologia, ormai entrata nella routine della ricerca genomica e della diagnostica. Inoltre da dati NGS è possibile ricostruire l'intera sequenza di genomi virali a DNA e RNA e di genomi batterici presenti nel campione e confrontarlo con i genomi di riferimento presenti nei database pubblici.

Di seguito si riporta per intero il paragrafo della linea guida WHO dedicato alle contaminazioni di DNA provenienti dalle linee cellulari immortalizzate:

Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks

Replacement of Annex 1 of WHO Technical Report Series, No. 878

5.2.2 Cellular DNA

The issue of rcDNA [residual cellular DNA] in biological products has been considered by many groups since the 1980s, and there has been an evolution of consensus on recommendations. The most recent WHO Recommendation (WHO Technical Report Series, No. 878) (1) sets the upper limit of rcDNA at 10 ng per parenteral dose. As stated below, while this value has proved helpful in the past, it does not take into consideration important factors such as the size of the DNA fragments and any potentially inactivating steps in the manufacturing process. Thus, it is important to take into consideration not only the limit of 10 ng per parenteral dose but other factors as well, when determining the acceptable level of rcDNA.

PCCs [Primary cell cultures] and DCLs [Diploid cell lines] have been used successfully for many years for the production of viral vaccines, and the rcDNA deriving from these cells has not been (and is not) considered to pose any significant risk. However, with the use of CCLs, which have an apparently indefinite lifespan, presumably due to the dysregulation of genes that control growth, and with the ongoing development of products from cells that are tumorigenic or were derived from tumours, the DNA from such cells has been considered to have the theoretical potential to confer the capacity for unregulated cell growth, and perhaps oncogenic activity, upon some cells of a recipient of the biological product.

Although the risk of such DNA has been estimated on the basis of certain assumptions and some experimental data, assessing the actual risk of such DNA has not been possible until recently, when preliminary data generated from new experimental systems began to quantify the risk (38). The potential risk of DNA arises from both of its biological activities, namely infectivity and oncogenicity. Infectivity could be due to the presence of an infectious viral genome in the cellular DNA of the cell substrate (39– 41). The viral genome could be that of a DNA virus, whether integrated or extrachromosomal, or of a proviral genome of a retrovirus. Both types of viral DNA have been shown to be infectious in vitro and, in several cases, in vivo (39, 40). The oncogenic activity of DNA could arise through its capacity to induce a normal cell to become transformed and perhaps to become tumorigenic. The major mechanism through which this could occur would be the introduction of an active dominant oncogene (e.g. myc, activated ras), since such dominant oncogenes could directly transform a normal cell. Other mechanisms would require that the rcDNA transforms through insertional mutagenesis, and have been considered less likely since the frequency of integration of DNA is generally low (42). The frequency of integration at an appropriate site, such as inactivating a tumour suppressor gene or activating a proto-oncogene, would be correspondingly lower (32).

³ <https://www.illumina.com/science/technology/next-generation-sequencing.html>
<https://www.atdbio.com/content/58/Next-generation-sequencing>

The 1986 WHO Study Group addressed the risk posed by the oncogenic activity of rcDNA in biological products for human use (12). Risk assessment based on a viral oncogene in an animal model suggested that in vivo exposure to 1 ng of rcDNA, where 100 copies of an activated oncogene were present in the genome, would give rise to a transformational event once in 10^9 recipients (27).⁴ On the basis of this and other evidence available at that time, the Study Group concluded in 1986 that the risk associated with rcDNA in a product is negligible when the amount of such DNA is 100 pg or less per parenteral dose. On the basis of a review of more recent data, those requirements were revised in 1998 to raise the acceptable level of rcDNA to 10 ng per dose.⁵

Studies in mice using cloned cellular oncogenes also suggest that the risk of neoplastic transformation by cellular DNA is probably very low (34, 43). However, more recent data have shown that cloned cellular oncogene DNA can induce tumours in selected strains of mice at levels below 1ng. In addition, single oncogenes can also be biologically active (44) and can initiate the tumour induction process. Because of these data and the recent evidence that genes encoding for certain micro-RNA species can be oncogenic in vitro (45–48), thus increasing the number of potential dominant cellular oncogenes, the oncogenic risk of DNA needs to be taken into account when tumorigenic cells are considered for use in the production of biologicals. This would be especially important for live attenuated viral vaccines where chemical inactivation of the DNA is not possible and where the only way to reduce the biological activity of DNA would be by nuclease digestion and the reduction in the quantity of DNA.

In addition to its oncogenic activity, the infectivity of DNA should be considered. Since a viral genome, once introduced, could amplify and produce many infectious particles, the infectivity risk is likely to be greater than the oncogenic risk. The polyoma virus genome is infectious in mice at about 50 pg (49), and a recent report demonstrated that 1 pg of a proviral copy of a retrovirus is infectious in vitro (50)⁶. Because such low levels of DNA may be biologically active, the amounts of rcDNA should be factored into safety evaluations when tumorigenic cell substrates are used, especially for live viral vaccines.

Consequently, considerations that need to be taken into account with respect to rcDNA are: (i) any reduction in the amount of the contaminating DNA during the manufacturing process; (ii) any size reduction of the contaminating DNA during the manufacturing process; and (iii) any chemical inactivation of the biological activity of contaminating DNA during the manufacturing process. A product might be considered by an NRA/NCL to have an acceptable level of risk associated with the DNA of the cell substrate on the basis of (i) and/or (ii) and/or (iii) when data demonstrate that appropriate levels have been achieved. For example, data have shown that nuclease digestion of DNA or chemical inactivation of DNA with beta-propiolactone, a virus-inactivating agent, can destroy the biological activity of DNA (38, 50, 51). Therefore, the use of these procedures may provide an additional level of confidence with respect to reduction of DNA risk.

For products such as monoclonal antibodies and subunit vaccines manufactured in tumorigenic cell substrates, it is necessary to demonstrate the clearance (removal and/or inactivation) of DNA by the manufacturing process. This may require validation of the main inactivating or removal steps. For example, data should be obtained on the effects of DNA-inactivating agents under specific manufacturing conditions, so that firm conclusions can be drawn on their DNA-inactivating potential for a given product.

There may be instances where CCL DNA is considered to pose a higher level of risk because it contains specific elements such as infectious retroviral proviral sequences. Under these circumstances, the steps taken to reduce the risks of rcDNA, such as reducing the size of DNA fragments, should be agreed in consultation with the NRA/NCL. (...)

Some products, especially certain live viral vaccines, are difficult to purify without a significant loss in potency, so the amount of rcDNA in those final products may be significantly higher than 10 ng per dose. Such cases are considered to be exceptional and should be discussed with the NRA/NCL.

⁴ Petricciani JC, Regan PJ. Risk of neoplastic transformation from cellular DNA: calculations using the oncogene model. *Developments in Biological Standardization*, 1986, 68:43–49.

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[Yang H1](#), [Zhang L](#), [Galinski M](#).

⁵ [Dev Biol Stand](#). 1998;93:136-8.

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⁶ Peden K et al. Biological activity of residual cell-substrate DNA. *Developmental Biology (Basel)*, 2006, 123:45–53

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[Sheng-Fowler L1](#), [Lewis AM Jr](#), [Peden K](#).

Per i seguenti vaccini l'analisi metagenomica è associata all'analisi del bianco e dello standard "Viral Multiplex Reference"

Analisi metagenomica su controllo negativo (bianco)

Risultati

La letteratura ^{1,2} riporta elenchi di genomi di microrganismi presenti nei laboratori di biologia molecolare, derivanti da kit di estrazione di DNA/RNA, da kit utilizzati per la retrotrascrizione, da vettori di clonaggio e da kit per la preparazione di librerie, oltreché da contaminazione ambientale, che creano un background di falsi positivi che deve essere filtrato dalle reads dei campioni portati avanti con la stessa procedura.

Di seguito una tabella con i genomi che si ritrovano nei nostri NTC e che vengono utilizzati per filtrare i campioni vaccinali prima dell'analisi Kraken. Molti coincidono con quelli già ritrovati in letteratura.

RNA Seq total frag	3233509			
Classification			n° fragments	% fragments
Bacteria	Proteobacteria	<i>Bejerinckia</i> , <i>Bosea</i> , <i>Bradyrhizobium</i> , <i>Brevundimonas</i> , <i>Caulobacter</i> , <i>Craurococcus</i> , <i>Devosia</i> , <i>Hoeftella</i> , <i>Mesorhizobium</i> , <i>Methylobacterium</i> , <i>Novosphingobium</i> , <i>Ochrobactrum</i> , <i>Paracoccus</i> , <i>Pedomicrobium</i> , <i>Phyllobacterium</i> , <i>Rhizobium</i> , <i>Roseomonas</i> , <i>Sphingobium</i> , <i>Sphingomonas</i> , <i>Sphingopyxis</i>	152201	5%
		<i>Comamonas</i> , <i>Cupriavidus</i> , <i>Curvibacter</i> , <i>Delftia</i> , <i>Duganella</i> , <i>Herbaspirillum</i> , <i>Janthinobacterium</i> , <i>Kingella</i> , <i>Leptothrix</i> , <i>Limnobacter</i> , <i>Masiella</i> , <i>Methylophilus</i> , <i>Methylovorax</i> , <i>Oxalobacter</i> , <i>Pelomonas</i> , <i>Polaromonas</i> , <i>Ralstonia</i> , <i>Schlegella</i> , <i>Sulfuritalea</i> , <i>Undibacterium</i> , <i>Variovorax</i>	79709	2%
		<i>Gamma-proteobacteria</i> : <i>Acinetobacter</i> , <i>Enhydrobacter</i> , <i>Enterobacter</i> , <i>Escherichia</i> , <i>Nevskia</i> , <i>Pseudomonas</i> , <i>Pseudoxanthomonas</i> , <i>Psychrobacter</i> , <i>Stenotrophomonas</i> , <i>Xanthomonas</i>	90745	3%
		<i>delta/epsilon</i> subdivisions	1990	0,06%
	Actinobacteria	<i>Aeromicrobium</i> , <i>Arthrobacter</i> , <i>Beutenbergia</i> , <i>Brevibacterium</i> , <i>Corynebacterium</i> , <i>Curtobacterium</i> , <i>Dietzia</i> , <i>Geodermatophilus</i> , <i>Janibacter</i> , <i>Kocuria</i> , <i>Microbacterium</i> , <i>Micrococcus</i> , <i>Microlunatus</i> , <i>Patulibacter</i> , <i>Propionibacterium</i> , <i>Rhodococcus</i> , <i>Tsukamurella</i>	1388647	43%
	Firmicutes	<i>Abiotrophia</i> , <i>Badilloa</i> , <i>Brevibacillus</i> , <i>Brochothrix</i> , <i>Facklamia</i> , <i>Paenibacillus</i> , <i>Streptococcus</i>	387929	12%
	Bacteroidetes	<i>Chryseobacterium</i> , <i>Dyadobacter</i> , <i>Flavobacterium</i> , <i>Hydrothalea</i> , <i>Niastella</i> , <i>Olivibacter</i> , <i>Pedobacter</i> , <i>Wautersella</i>	99297	3%
	Deinococcus-Thermus	<i>Deinococcus</i>	42	0,001%
	Acidobacteria	<i>Predominantly unclassified Acidobacteria</i> <i>Gp2</i> organisms	38	0,001%
Unassigned Bacteria			14764	0,5%
Eukaryotic			267004	8%
Unassigned cellular			50577	2%
Viruses		<i>Equine infectious anemia virus</i>	2854	0,10%
		<i>Pavlovirus</i>	376	0,01%
		<i>Bunyavirales</i>	14	0,0004%
		<i>Baculoviridae</i>	567	0,02%
		<i>Chordopoxvirinae</i>	2	0,0001%
		<i>Cafeteria roenbergensis virus</i> <i>CRV-1</i>	0	0,0000%
		<i>Herpesviridae</i> (<i>Chimpanzee herpesvirus strain 105640</i>)	9	0,0003%
		<i>Glypta fumiferanae ichnovirus</i>	566	0,02%
			841	0,03%
Archaea				
			331952	10%
Synthetic construct			363384	11%

Riferimenti bibliografici

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Metagenomic analysis on commercial product

Viral Multiplex Reference (NIBSC code: 11-242-xxx)

Descrizione prodotto

Il reagente viral multiplex 11/242-xxx, acquistato presso il **National Institute for Biological Standards and Control (NIBSC, UK)**, è destinato all'uso come riferimento in saggi di rilevazione di virus che impiegano tecnologie di sequenziamento ad elevata profondità (deep sequencing).

Il mix virale contiene 25 virus umani appartenenti a diverse famiglie di virus, con diverso tipo di struttura (con e senza envelope), tipo (a DNA o RNA) e dimensione del genoma e isolati da colture cellulari, campioni clinici o dopo passaggio in uova di pollo (Vedi Tab. 1 per dettagli). Questa mix virale è stata già utilizzata da diversi laboratori ^{1,2} che hanno anche svolto una analisi Real-Time PCR (vedi Ct values in Tab.1) che ha mostrato la minore capacità di quest'ultima tecnologia, rispetto all'NGS, di riconoscere la presenza di alcuni virus (in particolare nel reagente viral multiplex non sono rilevabili mediante Real-Time Norovirus GI e GII, Adenovirus 41, Coronavirus 229E, Influenza A H3N2 e B).

Virus composition of multiplex reagent 11/242-001.						
Group	Family	Envelope	Species/serotype	Genome size (kb)	PCR Ct value	Sample origin
dsDNA	Adenoviridae	No	Adenovirus 2	35,9	29,71	293 cell culture
			Adenovirus 41	34,2	ND	Clinical specimen
	Herpesviridae	Yes	Human herpesvirus 1	151,2	30,59	MRC5 cell culture
			Human herpesvirus 2	154,7	32,48	MRC5 cell culture
			Human herpesvirus 3 (VZV)	124,8	29,02	MeWo cell culture
			Human herpesvirus 4 (EBV)	171,7	31,27	B95-8 cell culture
			Human herpesvirus 5 (CMV)	233,7	28,95	MRC5 cell culture
dsRNA	Reoviridae	No	Rotavirus A	18,5	24,49	Clinical specimen
ssRNA (+)	Astroviridae	No	Astrovirus	6,8	30,53	Clinical specimen
	Caliciviridae	No	Norovirus GI	7,6	ND	Clinical specimen
			Norovirus GII	7,5	ND	Clinical specimen
			Sapovirus C12	7,5	33,37	Clinical specimen
	Coronaviridae	Yes	Coronavirus 229E	27,2	ND	MRC5 cell culture
	Picornaviridae	No	Coxsackievirus B4	7,4	30,72	Hep-2 cell culture
			Rhinovirus A39	7,1	31,16	MRC5 cell culture
			Parechovirus 3	7,2	29,35	LLC-MK2 cell culture
ssRNA (-)	Orthomyxoviridae	Yes	Influenza A virus H1N1	13,2	32,02	Egg passage
			Influenza A virus H3N2	13,6	ND	Egg passage
			Influenza B virus	14,2	ND	Egg passage
	Paramyxoviridae	Yes	Metapneumovirus A	13,3	31,86	LLC-MK2 cell culture
			Parainfluenzavirus 1	15,5	34,43	PRF5 cell culture
			Parainfluenzavirus 2	15,7	33,87	PRF5 cell culture
			Parainfluenzavirus 3	15,4	ND	PRF5 cell culture
			Parainfluenzavirus 4	17,4	31,83	PRF5 cell culture
			Respiratory syncytial virus A2	15,2	34,33	Hep-2 cell culture

Tab. 1. Da Mee ET, Preston MD, Minor PD, Schepelmann S, Participants CS. Development of a candidate reference material for adventitious virus detection in vaccine and biologicals manufacturing by deep sequencing. Vaccine. 2016;34(17):2035–43

Risultati

Di seguito i risultati dei due tipi analisi bioinformatiche svolte. La presenza di ciascun virus dell'elenco nella colonna 'Viral mix', è espressa sia in termini di numero assoluto che di percentuale sul totale dei frammenti rilevati dall'analisi svolta con il software Kraken. L'esito del secondo tipo di analisi (CLC assembly de novo) è rappresentato invece con un '+' o un '-', a seconda che siano stati trovati contigs (pezzi contigui di genoma) relativi a quel particolare virus. E' riportata solo l'analisi della libreria RNA-seq, poiché tutti i virus a dsDNA (primi 7 della lista) sono stati rilevati anche in questa libreria e non solo nella libreria DNA-seq.

Il resto dei frammenti sequenziati è stato classificato come 'unassigned' - 'mis-assigned' - 'lab contamination' - 'mammalian' - 'Saccharomyces' (percentuali non riportate). In questo mix virale è stata rilevata un consistente background costituito da DNA umano, di altri mammiferi e di lievito, derivante da cellule da cui i virus sono stati isolati.

Viral mix	CLC RNA	Kraken2	
		n° frammenti	% frammenti
Human adenovirus 2 (Human mastadenovirus C)	+	3934	0,05%
Human adenovirus 41 (Human mastadenovirus F)	+	25	0,0003%
Human Herpesvirus 1	-	9	0,0001%
Human alphaherpesvirus 2	+	45	0,0005%
Human alphaherpesvirus 3 (Herpesvirus 3 (VZV))	+	3139	0,04%
Human gammaherpesvirus 4	+	512	0,01%
Human herpesvirus 5	+	920	0,01%
Human rotavirus A	+	14370	0,2%
Human astrovirus 1	+	2180	0,02%
Norovirus GI	-	-	0%
Norovirus GII	-	6	0,00007%
Sapovirus	+	6035	0,07%
Coronavirus 229E	-	-	0%
Coxsackievirus B4	+	1864	0,02%
Human rhinovirus A39	+	3399	0,04%
Human parechovirus 3	+	764307	9%
Influenza A virus (H1N1)	+	77	0,0009%
Influenza A virus (H3N2)	-	7	0,00008%
Influenza B virus	-	8	0,00009%
Human metapneumovirus A	+	20373	0,2%
Human respirovirus 1 (parainfluenza virus 1 (HPIV-1))	+	8479	0,1%
Human rubulavirus 2 (parainfluenza virus 1 (HPIV-2))	+	855	0,01%
Human parainfluenza virus 3	+	1276	0,01%
Human parainfluenza virus 4	+	12773	0,1%
Human respiratory syncytial virus strain A2	+	212	0,002%
Non target viruses			
Equine infectious anemia virus complete genome	+	477	0,01%
Saccharomyces cerevisiae navavirus 20S RNA <W>	+	16	0,0002%
Bovine viral diarrhea virus 1	+	23686	0,3%
Enteroviruses	+	6500	0,07%
Bovine alphaherpesvirus 5	+	928	0,01%
Pestivirus	-	24358	0,3%

Dei 25 virus attesi nel reagente, sono stati rilevati 23 virus, con entrambi o con uno solo dei due metodi di analisi bioinformatica utilizzati.

Nello studio 2, è stato effettuato il sequenziamento dello stesso reagente 'viral mix' in 18 laboratori diversi e con metodiche di preparazione di librerie, sequenziamento NGS e bioinformatiche diverse.

I 2 virus non rilevati (Norovirus GII e Coronavirus 229E) non sono stati rilevati in questo studio multicentrico mediante Real-Time e non sono stati trovati con NGS in 9 su 18 laboratori. La maggior parte dei laboratori (11 su 18) non è stata in grado di rilevare almeno 3 target virali.

Nel reagente sono stati rilevati anche virus aggiuntivi (non target virus, cioè non dichiarati da NIBSC), alcuni dei quali (es. Bovine diarrhea virus, Enterovirus) già precedentemente identificati in questo mix ¹ da altri laboratori.

La possibile causa di presenza di ulteriori virus nel reagente è probabilmente dovuta all'isolamento di questi virus assieme a virus target, a partire da campioni clinici umani, alla propagazione anche di altri virus nelle colture cellulari utilizzate e all'aggiunta di siero fetale bovino al reagente.

Riferimenti bibliografici

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Analisi metagenomica su prodotto commerciale 20 Strain Staggered Mix Genomic Material (ATCC® MSA-1003™)

Il reagente 20 Strain Staggered Mix Genomic Material, acquistato presso ATCC (UK), è una 'mock community' ovvero una 'finta' comunità microbica che imita un campione metagenomico misto. Questo prodotto comprende DNA genomico preparato a partire da ceppi batterici sequenziati per intero, caratterizzati e autenticati ATCC (ATCC Genuine Cultures). Questo standard è stato utilizzato come verifica della procedura di laboratorio e di analisi. L'analisi metagenomica di questo standard ha fornito i seguenti risultati:

DNA Seq			
Classification	n° frammenti	% frammenti	% dichiarata da ATCC
Acinetobacter baumannii	10735	0,2%	0,18%
Actinomyces odontolyticus	2	0,00004%	0,18%
Bacillus cereus	176327	3,5%	1,8%
Bacteroides vulgatus	1088	0,02%	0,02%
Bifidobacterium adolescentis	489	0,01%	0,02%
Clostridium beijerinckii	123609	2,5%	1,8%
Cutibacterium acnes	6528	0,13%	0,18%
Deinococcus radiodurans	745	0,02%	0,02%
Enterococcus faecalis	704	0,01%	0,02%
Escherichia coli	929837	19%	18%
Helicobacter pylori	4738	0,1%	0,18%
Lactobacillus gasseri	4491	0,1%	0,18%
Neisseria meningitidis	9820	0,19%	0,18%
Porphyromonas gingivalis	578294	12%	18%
Pseudomonas aeruginosa	152307	3%	1,8%
Rhodobacter sphaeroides	1135927	23%	18%
Staphylococcus aureus	72598	1,5%	1,8%
Staphylococcus epidermidis	634940	13%	18%
Streptococcus agalactiae	31622	0,6%	1,8%
Streptococcus mutans	526420	11%	18%

Unassigned	14248	0,3%	0%
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Analisi bioinformatica

Pulizia delle sequenze (trimming)

Le sequenze degli adattatori e delle basi di DNA lette a bassa qualità sono state rimosse usando i software ERNE¹ e Cutadapt².

Filtraggio delle sequenze sul controllo negativo (bianco).

Al fine di eliminare eventuali contaminazioni dovute a reagenti di normale utilizzo in biologia molecolare (da kit di estrazione di DNA, di retrotrascrizione e di preparazione di librerie), le sequenze ottenute sono state filtrate su un database di genomi/sequenze rilevabili nel bianco (vedi report del controllo negativo).

Identificazione degli organismi di origine delle sequenze di DNA e cDNA/RNA con il software Kraken.

L'analisi metagenomica è stata eseguita utilizzando il software Kraken³ sul database 'Human-Virus-Bacteria_25mer' (<https://ccb.jhu.edu/software/kraken/>). Kraken è un classificatore che assegna etichette tassonomiche a brevi letture del DNA. Lo fa esaminando i k-mers all'interno di una lettura e interrogando un database con quei k-mers.

Le sequenze classificate con software Kraken sono state confermate manualmente con il software BLAST (Basic Local Alignment Search Tool, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Riferimenti bibliografici

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2. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet journal, [S.l.], 17 (1): 10-12 (2011). ISSN 2226-6089. Date accessed: 02 Apr. 2015. doi:<http://dx.doi.org/10.14806/ej.17.1.200> paper
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Dossier EMA – NGS Priorix Tetra

Nome prodotto: Priorix Tetra

Lotti analizzati: lot. A71CB205A e lot. A71CB256A

Tipo di prodotto: Vaccino tetravalente morbillo, parotite, rosolia, varicella

Produttore: GlaxoSmithKline, Belgio

Composizione (da bugiardino): virus vivi attenuati: 1) Morbillo (ssRNA) ceppo Schwarz, coltivato in colture di cellule embrionali di pollo; Parotite (ssRNA) ceppo RIT 4385, derivato dal ceppo Jeryl Lynn, coltivato in colture di cellule embrionali di pollo; Rosolia (ssRNA) ceppo Wistar RA 27/3, coltivato in cellule diploidi umane (MRC-5); Varicella (dsDNA) ceppo OKA coltivato in cellule diploidi umane (MRC-5).

Analisi del DNA:

La misurazione della concentrazione del DNA con fluorimetro QuBit ha evidenziato che il lotto A71CB205A, contiene una quantità di gDNA di 1.7 µg totali per dose da 0.5mL, così calcolata:

9.41ng/µl (concentrazione determinata al QuBit) x 45 (volume di risospensione finale del DNA dopo estrazione, espresso in microlitri) x 4 (il volume di partenza sottoposto alla procedura di estrazione è ¼ del volume della dose contenuta nell'intera fiala pari a 0.5mL).

La misurazione della concentrazione del DNA con fluorimetro QuBit ha evidenziato che il lotto A71CB256A, contiene una quantità di gDNA di 3.7 µg totali per dose da 0.5mL, così calcolata:

40.8 ng/µl (concentrazione determinata al QuBit) x 55 (volume di risospensione finale del DNA dopo estrazione espresso in microlitri) x 5/3 (il volume di partenza sottoposto alla procedura di estrazione è sto di 300 µl su 500 µl di sospensione).

Il DNA umano trovato in questo lotto è in rapporto relativo di circa 8 a 1 rispetto al DNA della varicella (vedi risultati seguenti della classificazione dei frammenti DNA-seq, in cui emerge che l'88% del totale dei frammenti di DNA sequenziati è di origine umana, e l'11% è di genoma di virus della varicella). Considerando che l'NGS è una tecnologia quantitativa, la quantificazione fluorimetrica del DNA totale estratto dal vaccino (es. lot. A71CB256A = 3,7 microgrammi per dose), associata alla considerazione di quantificazione relativa fatta sopra (8:1), ci permette di poter dire che il DNA umano potrebbe essere di **circa 2,9 microgrammi per dose**, rispetto a circa 740 nanogrammi di DNA della varicella. E' inoltre plausibile che **almeno una porzione del DNA ad alto peso molecolare che si vede su gel possa essere DNA umano ad alto peso molecolare**.

Analisi dell'RNA:

La quantità di RNA contenuta nella fiala di vaccino lotto A71CB256A è risultata essere di circa 200ng.

Il RIN pari a 8 indica un RNA di ottima qualità e un RNA eucariotico intatto, essendo presenti entrambi i picchi 18S e 28S tipici dell'RNA eucariotico.

Risultati delle analisi DNA-seq e RNA-seq sui due lotto eseguite con il software Kraken

Di seguito il risultato della classificazione tassonomica delle sequenze. La presenza di DNA e RNA è espressa in termini di numero e di percentuale sul totale dei frammenti prodotti, attribuiti dai database pubblici ai diversi organismi. Il resto dei frammenti ottenuti sono stati classificati come 'unassigned'-'mis-assigned' -'lab contamination' (percentuali non riportate).

Priorix Tetra lot. A71CB205A (giugno 2018) – analisi DNA

DNA Seq			
Classification		n° frammenti	% frammenti
Homininae (<i>Homo sapiens</i>)		2853788	74%
Viruses*		538112	14%
Aves (<i>Gallus gallus</i>)		152256	4%
*Viruses classification		n° frammenti	% frammenti
dsDNA viruses		538112	14%
	Human alphaherpesvirus 3	537849	14%
ssRNA viruses		34	0.01%
	Mumps rubulavirus	19	0.0005%
	Measles morbillivirus	12	0.0003%
	Rubella virus	3	0.00008%

Priorix Tetra lot. A71CB205A (giugno 2018) – analisi RNA

RNA Seq			
Classification		n° frammenti	% frammenti
Homininae (<i>Homo sapiens</i>)		9036993	87%
Viruses*		499098	5%
Aves (<i>Gallus gallus</i>)		16361	0.2%
*Viruses classification		n° frammenti	% frammenti
dsDNA viruses		497498	5%
	Human alphaherpesvirus 3	497465	5%
ssRNA viruses		1324	0.01%
	Mumps rubulavirus	874	0.008%
	Measles morbillivirus	441	0.004%
	Rubella virus	7	0.00007%

Priorix Tetra lot. A71CB256A (dicembre 2018) – analisi DNA

DNA Seq			
Classification		n° frammenti	% frammenti
Homininae (<i>Homo sapiens</i>)		5150674	88%
Viruses*		643575	11%
*Viruses classification		n° frammenti	% frammenti

dsDNA viruses		643549	11%
	Human alphaherpesvirus 3	643542	11%

Priorix Tetra lot A71CB256A (dicembre 2018) – analisi RNA

RNA Seq			
Classification		n° frammenti	% frammenti
Homininae (<i>Homo sapiens</i>)		4210032	68%
Viruses*		419863	7%
*Viruses classification		n° frammenti	% frammenti
dsDNA viruses		418104	7%
	Human alphaherpesvirus 3	417868	7%
ssRNA viruses		845	0.01%
	Mumps rubulavirus	508	0.008%
	Measles morbillivirus	217	0.004%
	Rubella virus	0	0

Primi approfondimenti sul DNA umano contenuto in Priorix Tetra

L'evidenza diretta che dentro questo prodotto ci sia un genoma umano **COMPLETO** (cioè con geni e sequenze non codificanti), ad alto peso molecolare (vedi PFGE) e/o frammentato, è data dal risultato dell'allineamento delle reads di derivazione umana (il 70-80% del dataset nei tre lotti testati, di cui il primo lotto è stato sequenziato nel 2017, ma non sono stati presentati i dati in questo report) sul riferimento umano UCSC hg19, effettuato con un software standard (BWA) utilizzato dalla comunità scientifica per allineare sequenze NGS su genomi di riferimento (Bioinformatics, Volume 25, Issue 14, 15 July 2009. Fast and accurate short read alignment with Burrows–Wheeler transform).

Nella seguente tabella, evidenziato in arancione, è riportato il risultato espresso in 'Av_cov = copertura media' dell'allineamento delle sequenze umane dei 3 lotti di Priorix tetra testati (1st-2nd e 3rd lot) sui cromosomi umani. Nella colonna 1, chM è il DNA mitocondriale, mentre Ch1 fino a ChY sono i cromosomi umani, inclusi i cromosomi sessuali X e Y. Nella colonna 2 è riportata la lunghezza dei cromosomi umani assemblati espressa in paia di basi.

La copertura è bassa (Avg_cov in media lungo ogni cromosoma < di 1x) ma l'omogeneità di distribuzione delle reads che si allineano in modo univoco, lungo tutti i cromosomi umani e la presenza anche di reads che si allineano a più alta copertura sul genoma mitocondriale, permette di riconoscere indiscutibilmente una situazione simile ad un low-pass genome sequencing di un genoma umano individuale. Per una più facile comprensione di quanto affermato, nella parte indicata in verde sono riportati dei low pass whole genome sequencing (5 campioni, 4 femmine e 1 maschio) umani a circa 10 milioni di reads, cioè una profondità analoga a quella prodotta per i 3 lotti vaccinali di Priorix Tetra.

Dal rapporto tra la copertura media per il cromosoma X e Y (indicati in rosso nell'ultima riga della tabella) emerge anche l'indicazione del sesso dell'individuo (maschile).

Il DNA umano contenuto nei lotti di Priorix ad ora sequenziati, è stato anche qualificato come appartenente alla linea fetale MRC-5 cioè la linea cellulare continua derivata da tessuto polmonare di feto abortivo maschile degli anni '60, in cui vengono fatti crescere il virus della varicella e della rosolia. L'analisi delle varianti del DNA mitocondriale presente nel vaccino rispetto al DNA mitocondriale della linea MRC-5 (il DNA della linea cellulare è stato acquistato presso ATCC) ha evidenziato che si tratta dello stesso 'individuo', poiché non è presente alcuna variante genetica.

Discussione

Di seguito si riportano degli estratti delle linee guida internazionali che normano la qualità dei vaccini contenenti virus attenuati:

<https://www.who.int/biologicals/Molecular%20Methods%20Final%20Mtg%20Report%20April2005.pdf?ua=1>

Report – WHO informal consultation on the application of molecular methods to assure the quality, safety and efficacy of vaccines – WHO 7-8 april 2005

1. Quantitative mutant analysis of viral quasispecies by MAPREC test and MALDI- TOF mass spectrometry. Dr K. Chumakov
In the QC of live attenuated viral vaccines, genetic stability is of great concern. The presence of even small quantities of mutants or revertants may indicate incomplete or unstable attenuation that may influence vaccine safety. Oral poliovirus vaccine can be monitored with the use of 'mutant analysis by polymerase chain reaction (PCR) and restriction enzyme cleavage' (MAPREC). Genetic variation in live attenuated mumps virus vaccine has been investigated by using both MAPREC and a platform (DNA massarray) based on matrix-assisted laser desorption/ionization time-of-flight (MALDI- TOF) mass spectrometry. Mumps vaccines prepared from the Jeryl Lynn (JL) strain typically contain at least two distinct viral substrains, JL1 and JL2, which have been characterized by full-length sequencing. Dr Chumakov reported the development of assays for characterizing sequence variants in these substrains and demonstrated their use in quantitative analysis of substrains and sequence variations in mixed virus cultures and mumps vaccines. The results obtained from both the MAPREC and MALDI-TOF methods showed excellent correlation. The result suggested the potential utility of MALDI-TOF for routine QC of live viral vaccines and for assessment of genetic stability and quantitative monitoring of genetic changes in other RNA viruses of clinical interest. The molecular tests mostly provide a gauge of consistency, and quantification of mutants by MAPREC, MALDI-TOF or microarray hybridization can be used as a surrogate test for viral vaccines. For the future, there is a need for a method to look at the entire genome and be able to detect mutational heterogeneities.

https://www.ema.europa.eu/en/documents/scientific-guideline/position-statement-dna-host-cell-proteins-hcp-impurities-routine-testing-versus-validation-studies_en.pdf

The European Agency for the Evaluation of Medicinal Products

Human Medicines Evaluation Unit

London, 10 June 1997 CPMP/BWP/382/97

CPMP position statement on DNA and host cell proteins (HCP) impurities, routine testing versus validation studies

RESIDUAL HOST CELL DNA

Regarding residual DNA, it is already accepted for bacteria- and yeast- derived products that there is no need for routine testing provided that acceptable levels in the final product are achieved and, adequate validation data are submitted in the dossier. As far as DNA from continuous mammalian cell lines (CCLs) is concerned, this impurity was considered, in the past, as a risk factor because of concerns that residual host DNA may be tumorigenic. Further information, however, now suggests that CCL DNA poses much less of a risk than previously thought and accordingly should be considered as a general impurity (WHO Expert Committee on Biological Standardisation: Highlights of the 46th meeting, October 1996, in WHO Weekly Epidemiological Record, 1997, 72, 141-145).

Validation studies (e.g. spiking experiments using an adequate size distribution of DNA) should be performed in an attempt to identify the major steps capable of reducing the DNA burden and to document the capacity of those steps in reducing residual cellular DNA content in the final product, to an acceptable and defined level. The technology for DNA quantitation is now well defined and reproducible.

In addition to the validation studies, results of DNA quantitation on a minimum number of production batches (e.g. 5 consecutive batches) should be provided to demonstrate the reproducibility of the production process in reducing residual DNA to the level expected from the validation studies. Based on satisfactory validation data and consistent results on a limited number of production batches, it seems reasonable not to perform routinely CCL DNA tests at the purified bulk level (or other appropriate steps).

In some cases (e.g. previously non-approved CCLs, transforming DNA sequences from viral vectors, etc.) it may be necessary to routinely control the elimination of DNA.

As far as products derived from CCLs already authorised are concerned, it could be acceptable that routine DNA testing be discontinued. However, this will be subject to a formal variation of the Marketing Authorisation. The variation documentation should provide satisfactory validation data, along with results gained by the applicant since the beginning of the production to establish consistency of the process, in terms of residual DNA level.

https://www.who.int/biologicals/publications/trs/areas/vaccines/mmr/WHO_TRS_840_A3.pdf?ua=1

Annex 3 Requirements for measles, mumps and rubella vaccines and combined vaccine (live) (Requirements for Biological Substances No. 47) WHO

Appendix 2 Requirements for human diploid cells used for the production of measles, mumps and rubella vaccines (live)

Source materials

The cell seed and MWCB (manufacturer's working cell bank) shall be approved by and registered with the national control authority. The cells shall have been characterized with respect to their genealogy, growth characteristics, genetic markers (HLA), viability during storage and karyology, and have been shown to be free from bacteria, mycoplasmas, fungi and haemadsorbing and other viruses by the relevant tests in these Requirements. In addition, the cells of the MWCB shall have been shown to be diploid and stable with respect to karyology and morphology by the tests outlined in this section. The MWCB shall also have been shown to yield cell cultures capable of producing vaccine that is both safe and immunogenic in humans. Each production cell culture shall consist of cells at a passage level of up to two-thirds of the life span of the accepted cell strain and shall be tested for identity. It shall comply with the tests outlined in sections 2.1.1, 2.1.2 and 2.1.4 for normal karyology and freedom from adventitious agents.

Other tests for extraneous agents

Suitable tests approved by the national control authority shall be performed to exclude the presence of retroviruses and the integration of nucleic acid of viral origin (hepatitis B virus and human immunodeficiency virus) in the genome of MWCB cells. In some countries, the cells are also examined in ultra-thin sections and by negative staining under the electron microscope.

2.1.3 Freedom from tumorigenicity

The cells of the MWCB shall be shown to be free from potential tumorigenicity by appropriate animal tests, including positive controls, approved by the national control authority. Suitable tests in immunosuppressed animals are as follows.

2.1.4 Chromosomal characterization of the cell seed

At least four samples from the cell seed shall be examined as described in section 2.1.5 at approximately equal intervals over the life span of the cell line during serial cultivation. Each sample shall consist of 1000 metaphase cells. It is recommended that photographic reconstruction should be employed to prepare chromosome-banded karyotypes of 50 metaphase cells per 1000-cell sample, by either G-banding or Q-banding techniques. The incidence of karyotypic abnormalities (pseudodiploidy, inversions, translocations, etc.) that are detectable with the greater resolution provided by banding should not exceed that approved by the national control laboratory.

2.1.5 Chromosomal characterization of the MWCB

For the determination of the general character of the MWCB, a minimum of 500 cells in metaphase shall be examined at the production level, or at any passage thereafter, for frequency of poliploidy and for exact counts of chromosomes, frequency of breaks, structural abnormalities, and other abnormalities such as despiralization or marked attenuations of the primary or secondary constriction. The cells of the MWCB shall have normal karyology. For vaccine production, examination of the cells is usually made between the 27th and 33rd population doubling. The national control authority should determine the permissible level of cell population doubling. For WI-38 and MRC5 cells examined in metaphase, the generally accepted upper limits for abnormalities in 1000- and 500-cell samples are:¹

<i>Abnormality</i>	<i>1000 cells</i>	<i>500 cells</i>
Chromatid and chromosome breaks	47/1000	26/500
Structural abnormalities	17/1000	10/500
Hyperploidy	8/1000	5/500
Hypoploidy	180/1000	90/500
Polyploidy	30/1000	17/500

All cells showing abnormalities shall be subjected to detailed examination and records shall be maintained of the detailed criteria applied to particular abnormalities in the karyotype analysis. Stained slide preparations of the chromosomal monitoring of the working cell bank, or photographs of these, shall be maintained permanently as part of the record of the MWCB.

<https://www.fda.gov/media/78428/download>

Guidance for Industry

Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications

U.S. Department of Health and Human Services Food and Drug Administration Center for Biologics Evaluation and Research [February 2010]

A. TESTING FOR ADVENTITIOUS AGENTS Assurance that products are free of adventitious agents is a critical component of meeting the 21 CFR 610.13 requirement for purity. Cultures must be tested for the presence of detectable microbial agents and tests necessary to assure the safety and purity of the product may be required (21 CFR 610.18). Your biological starting materials should be characterized sufficiently to ensure that they do not contaminate the final product with extraneous infectious organisms, such as bacteria, fungi, cultivatable and non-cultivable mycoplasmas and spiroplasma, mycobacteria, viruses, and the agent(s) responsible for transmissible spongiform encephalopathies (TSEs). For a substance to be considered free of a contaminant, your assay should demonstrate, at a predefined level of sensitivity, that a certain quantity of the substance is free of that contaminant. Alternatively, a validated process that is known to remove a contaminant to a defined level may be used to demonstrate the absence of that contaminant. If an adventitious agent is known to be present in your cell substrate or viral seed, then you should demonstrate that your

production process is sufficiently robust to eliminate or inactivate the agent with an appropriate margin of safety. You should avoid exposure of your product to agents that are known to be infectious for humans (other than the vaccine virus) and/or to agents for which there are no appropriately sensitive validated testing procedures (such as TSEs). You should have appropriate and sufficient controls in place to assure that such exposures do not occur

2. In Vitro Tests for Viruses

Cell culture safety tests can detect a variety of adventitious viruses that include cytopathic viruses, hemadsorbing viruses, and hemagglutinating viruses.

· Human Diploid Cells Human viruses: adenoviruses, alphaviruses, coronaviruses, enteroviruses (including poliovirus, echovirus, and coxsackie B virus), flaviviruses, certain strains of hepatitis A virus, herpesviruses (including herpes simplex virus, varicella zoster virus, and cytomegalovirus), orthomyxoviruses, paramyxoviruses, rabies virus, reoviruses, some rhinoviruses, and rubella virus.

4. Testing for Genetic Stability You should demonstrate the genetic stability of your cell substrate from the establishment of the MCB through and perhaps beyond the end of production. For an engineered cell line, the inserted gene of interest should remain intact and at the same copy number, and be expressed at comparable levels throughout production. Also, a diploid cell strain should remain diploid throughout. If such characteristics are not stable, then you should demonstrate that the instability does not adversely impact manufacturing or product consistency. For methods to assess a cell substrate's genetic stability, reference is made to the ICH Q5B and Q5D documents (Refs. 30 and 3, respectively).

C. OTHER TESTS

1. Testing for the Presence of Residual Cells You should assure that your final vaccine product does not contain residual cells. Processes, such as filtration, should be implemented and validated to ensure that intact cells are not present in the final product. Validation that residual cell removal processes are robust is important for immortalized cells. Determining the extent to which intact cells (or other materials known to be smaller than intact cells) are cleared by these processes is an important part of this validation. 36 Contains Nonbinding Recommendations

2. Testing for Residual Cellular DNA Residual DNA might be a risk to your final product because of oncogenic and/or infectivity potential. There are several potential mechanisms by which residual DNA could be oncogenic, including the integration and expression of encoded oncogenes or insertional mutagenesis following DNA integration. Residual DNA also might be capable of transmitting viral infections if retroviral proviruses, integrated copies of DNA viruses, or extrachromosomal genomes are present. The risks of oncogenicity and infectivity of your cell-substrate DNA can be lessened by decreasing its biological activity. This can be accomplished by decreasing the amount of residual DNA and reducing the size of the DNA (e.g., by DNase treatment or other methods) to below the size of a functional gene (based on current evidence, approximately 200 base pairs). Chemical inactivation can decrease both the size and biological activity of DNA. If DNA removal, digestion, or inactivation is undertaken, you should validate your methods. You should measure the amount and size distribution of residual DNA in your final product. For widely used human diploid cell strains, such as MRC-5 and WI-38 cells, measurement of residual DNA might be unnecessary because we do not consider residual DNA from these human diploid cells to be a safety issue. We might require limitation of the amount of residual DNA, depending on the potential risks associated with that DNA, for human diploid or primary cell types for which there is less experience. You should limit residual DNA for continuous non-tumorigenic cells, such as low-passage Vero cells, to less than 10 ng/dose for parenteral inoculation as recommended by WHO (Ref. 31). Because orally administered DNA is taken up approximately 10,000-fold less efficiently than parenterally administered DNA, we recommend limiting DNA to less than 100 µg/dose for oral vaccines (Ref. 32). If you are using cells with tumorigenic phenotypes or other characteristics that give rise to special concerns, more stringent limitation of residual DNA quantities might be needed to assure product safety

Da quanto previsto dalle linee guida sopra riportate, la presenza di DNA fetale proveniente dalla linea cellulare MRC-5 e WI-38, in quanto diploidi, non prevede dei limiti superiori. La motivazione risiede nel fatto che tali linee non sono considerate tumorali perché con un ciclo replicativo finito. Tuttavia, si portano all'attenzione dell'EMA le recenti acquisizioni sulla capacità trasformante del DNA fetale e gli effetti nocivi dell'inevitabile contaminazione da HERVs presenti nei vaccini MPR ed MPRV.

Di seguito si riporta un documento gentilmente fornito dalla dr.ssa Theresa Deisher: ⁷

RESIDUAL CELL SUBSTRATE DNA.

The FDA and their scientific experts have been debating the dangers of residual cell substrate DNA in vaccines for over 50 years, without resolution or appropriate study (6) **(page 1 abstract)** (7) (page 7 line 15 - page 8 line 3-8, page 10 lines 9-13). In an FDA 1999 workshop, committee participants stated that "Thus, based on the data, it would appear that under some circumstances there could be an infectious risk from residual DNA. Several factors may influence an assessment of the tumorigenicity⁸ or infectivity risk associated with residual DNA. These include the total quantity of DNA in the vaccine, the number of doses to be given, the size of the DNA, sequence-related properties of the DNA, for example whether it encodes a virus, the number of copies of potentially infectious sequences per cell, and the state of the DNA, which I take to mean such factors as whether it is a cellular genome, whether it is linearized or circular, et cetera. For many of these kinds of considerations raised on the slide, although it is likely that they have an effect on the ultimate infectivity or tumorigenicity of the DNA, **these studies have not in general been performed in a quantitative fashion that would enable us to apply a quantitative risk assessment model.**" (emphasis added) (7) page 76 line 13 through page 77 line 8).

Residual cell substrate DNA is DNA in the final vaccine that is carried over from the manufacturing process. It is DNA from the cell line used to manufacture the virus for the vaccine. While the FDA and experts have been clearly aware of the potential for cell substrate DNA to integrate into the recipient's genome and cause mutations and genomic instability (8) page 9 section G "Theoretical concerns regarding DNA integration include the risk of tumorigenesis if insertion reduces the activity of a tumor suppressor or increases the activity of an oncogene. In addition, DNA integration may result in chromosomal instability through the induction of chromosomal breaks or rearrangements") they have unfortunately preferred to rely on the theories of experts rather than on empirical experiments, to determine the risk associated with manufacturing vaccines and biologics in human diploid cell lines (9) (page 31 "Primary and diploid cells have been used successfully and safely for many years for the production of viral vaccines, and the residual cellular DNA deriving from these cells has not been **(and is not) considered** to pose any risk." (Emphasis added). For instance, Dr. Krause stated during the 1999 FDA workshop on evolving scientific and regulatory perspectives on cell substrates for vaccine development that "To my way of thinking, these are the kinds of questions which need to be answered. The question is do they need to be answered on an intuitive basis or do they need to be answered on an experimental basis." (7) (page 87 lines 11-15). Clearly these questions had not been experimentally addressed before MMR II and Varicella were on the market.

In early guidance meetings, regulatory agencies and experts argued for a recommended limit of 10 pgs cell substrate DNA per dose (10 billionths of a gram) (10) (page 1 column 2, lines 29-31), which has subsequently been relaxed twice to 100 pgs (100 billionths of a gram) (9) (page 32 line 12) and then to 10 ngs (10 millionths of a gram) cell substrate DNA per dose (11) (page 15 section A4.3.7 line 5-6). Neither limit was based upon empirical study or data to justify the guidance (10) (p 1-2) (9) (page 32 lines 5-15) (11) (page 15 section A4.3.7). Particularly, ignoring the large body of scientific publications about species specific DNA integration for gene correction therapy, the FDA and experts have preferred to consider residual DNA from human diploid cell lines as 'safe' (9) (page 32 lines 22-23) (10), (12) (page 42 lines 5-7)⁹, and the guidelines have been primarily if not exclusively applied to what are called continuous cell lines and not the human diploid cell lines of concern for vaccine recipients (9) (page 31). However, the extremely high levels of cell substrate DNA residuals contained in the Varivax vaccine were recognized as a potential safety hazard (13) (page 3), and Merck did conduct additional safety studies. Unfortunately, those studies were not appropriate to definitively

⁷ <https://www.soundchoice.org/about-us/>

⁸ Capable of causing tumors

⁹ I note that to obtain a license to market a vaccine or any other drug, a manufacturer must establish that the product is safe. I am informed by counsel that the law is contained in 42 U.S.C.A. § 262 and in particular §(C)(i)(I). In my experience this requires the manufacturer to conduct and submit studies to satisfy the FDA of the safety of the product. I am informed by counsel that the FDA may only license a vaccine regulated by 21 CFR Parts 600 through 680 and indirectly by 21 CFR Parts 210 and 211 when all of the applicable requirements have been met. Vaccines must be proven to be safe and counsel informs me that this word is defined by 21 C.F.R. 300.3 (p) "The word safety means the relative freedom from harmful effect to persons affected, directly or indirectly, by a product when prudently administered, taking into consideration the character of the product in relation to the condition of the recipient at the time." Here the persons given vaccinations are largely well children and no risk is acceptable as the cost benefit analysis done by a drug manufacturer is limited to the patient's health.

determine the risk of the cell substrate DNA. Merck's Varivax Summary Basis for Approval states that "The approximately 2 micrograms of cellular DNA per dose of vaccine was determined to be unlikely to integrate into host cells and cause harm under the conditions of vaccination.", however, they did not appear to have done ANY experiments to determine the rate of insertion of the extremely high levels of residual DNA into human cell lines. Determination of tumorigenicity of the residual DNA in nude mice, as conducted by Merck, is not an appropriate safety measure, as 'wild' DNA inserts almost solely in a species specific manner, and therefore, it should not be surprising that they did not observe tumors in nude mice. Furthermore, other tests of residual DNA used to establish the so-called 'safety' of DNA residuals were conducted in non-human primates (9) (page 32), again, the safety assessments did not use human cells or cell lines which are needed to determine the insertional mutagenesis potential of residual HUMAN DNA fragments.

Experts participating in the FDA 1999 committee workshop concluded that DNA insertion was minimal to no risk. In particular, the FDA's Dr. Krause and Dr. Lewis, leaders of the 1999 FDA workshop, published a paper in 1998 stating that "Because the risk of such events is now perceived to be very low, the previously accepted limit of 100 pg per dose of DNA from continuous cell lines has been increased to 10 ng and a proposal has been made to consider DNA as an impurity, rather than a risk factor" and "Human diploid cell lines such as WI-38 and MRC-5 cells have no evidence of containing endogenous infectious DNA. Moreover, DNA from such cell lines appears to carry no definable risk of oncogenicity" (14). However, despite their earlier publications and their pronouncements at that 1999 meeting, these FDA experts did continue to work on methods that would reduce the risk of DNA insertional mutagenesis, as will be discussed in the next paragraph.

It is also perplexing that in 1999, the same year that Dr. Krause had declared DNA to be no significant risk to recipients of vaccines, he published a study stating "that the ability of a virus or a tissue injury to increase MHC gene expression is duplicated by any fragment of double-stranded (ds) DNA or dsRNA introduced into the cytoplasm of nonimmune cells", demonstrating the clear danger of autoimmune reactions from residual human diploid dsDNA¹⁰ in vaccines. (15). There was a clear conflict between his empirical results published in 1999 and his opinion given at the 1999 FDA safety review meeting that the phenomena he has demonstrated constitutes no risk.

Since the 1999 Krause publication, the ability of ss and dsDNA fragments to activate immune effector cells, through both Toll Like Receptor ("TLR")¹¹ dependent and independent activities, has been well documented (16). Despite his 1999 publication demonstrating the autoimmunity dangers of residual cell substrate DNA in vaccines, at the 1999 FDA committee meeting Dr. Krause states that "To my way of thinking these are the kinds of questions which need to be answered. The question is do they need to be answered on an intuitive basis or do they need to be answered on an experimental basis." (7) (page 87). Indeed, autoimmune disease is a likely response to the injection of residual fetal DNA in vaccines. Since the DNA is foreign to the child an immune response to the fetal DNA is highly likely. Unfortunately, while the DNA is foreign enough to elicit an immune response it is also similar enough to ultimately lead to a self-attack or an 'auto-immune' response. Indeed, genetic engineering has demonstrated the dangers of auto-immune responses in clinical trials. Patients treated with a genetically engineered thrombopoietin (TPO) molecule developed antibodies to the foreign TPO that cross-reacted and led to an auto-immune attack on their endogenous TPO and subsequent thrombocytopenia (17) (18). As a result, development of homologous genetically engineered TPO agonists was suspended. Since the first submission of this report, independent publications have documented the presence of auto-immune anti-dsDNA, anti-ANA, and anti-neuron antibodies in serum of autistic children but not age and sex matched controls (Mostafa 2014 ref 18b, AL-Ayadhi 2014 ref 18c, Mostafa 2015 ref 18d and Mostafa ref 18e and Piras ref 18f and Anderson ref 18g).

Another participant of the 1999 FDA committee meeting that intuitively determined that human diploid cell contaminants posed no risk to the vaccine recipients was Dr. Onions. However, after the 1999 workshop, Dr. Onions began pursuing more sensitive techniques to determine vaccine safety and later published a 2011 paper on methods of enhanced sensitivity to detect adventitious viral contaminants in vaccines (19). Dr. Onions 2011 paper describes a technique called massive parallel sequencing that can detect novel or latent viruses in manufacturing cell lines and lauds this technique as an "unbiased and comprehensive method to identify viruses and other adventitious agents, without prior knowledge of the nature of those agents." (19) (page 1 Introduction column 1 lines 7-10).

In 2010, Victoria et.al. published a study revealing the presence of viral contaminants in various vaccines that had previously been undetected, including HERVK retrovirus in Varivax and MMRII (20) (p 6036, 6037). This provided direct evidence that Dr. Krause's 1998 statement about the

¹⁰ Double Stranded DNA.

¹¹ Toll-like receptors (TLRs) recognize distinct pathogen-associated molecular patterns and play a critical role in innate immune responses. They participate in the first line of defense against invading pathogens and play a significant role in inflammation, immune cell regulation, survival, and proliferation., Barton GM, Kagan JC (2009) A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nat. Rev. Immunol.* 9(8), 535-42.

absence of viral contamination of human diploid cell lines was incorrect. Perhaps even more intriguing are three 2011 publications from the same Dr. Krause that describe methods of enhanced sensitivity to detect contaminating infectious DNA in vaccines. It would appear that by 2011 he understood that his 1999 statement that such contamination was not an issue was incorrect (21) (22) (23).

It would appear that by 2011, at the latest, the FDA scientists and experts who theorized in 1999 that human diploid residuals in vaccines were safe had found that research conducted in their own laboratories has rendered their opinions unsupportable. In the face of that fact it is strange indeed that the respondent has adamantly refused to conduct the after-market tests made possible by the existence of the Vaccine Safety Data Link to determine if these now well established hazards are responsible for the autism epidemic.

THE CONTAMINANTS IN THE VACCINES

MMR II

The rubella portion of the MMRII vaccine (Meruvax) is manufactured using the human diploid cell line WI-38 and the Meruvax is contaminated with greater than 150 nanograms cell substrate dsDNA and ssDNA per dose, fragmented to approximately 215 base pairs in length (MMR II package insert attached and Deisher data, submitted for publication attached "Environmental Triggers and AD submitted manuscript"). 150 ngs of DNA is equivalent to the total amount of DNA in over 22,000 cells, and is approximately 1.5 trillion fragments. Additionally, the MMRII vaccine is contaminated with fragments of the HERVK retrovirus (20).

VARIVAX

The Varivax (chickenpox) vaccine is manufactured using the human diploid cell line MRC5, and is contaminated with 2 micrograms of cell substrate double stranded (ds) DNA (13) (page 3 line 21). Single stranded (ss) DNA levels are not reported in Merck's Varivax Summary Basis for Approval document nor is the length of the DNA fragments contaminating the vaccine. The residual DNA is largely fragmented and the approximate number of fragment pieces is estimated to be up to 1 trillion in the Varivax vaccine. This level of contamination provides a significant number of opportunities for DNA fragment integration into the recipient's genome as will be discussed in further detail. Varivax is also contaminated with fragments of the HERVK retrovirus (20) (p.6037) due to culturing of the varicella virus using the WI-38 human diploid cell line at one point.

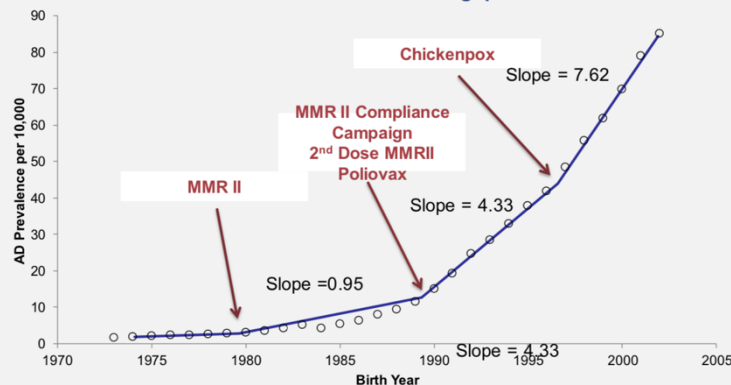
HAVRIX

Havrix, a hepatitis A vaccine, is manufactured using the human diploid cell line MRC5 and is contaminated with more than 300 nanograms cell substrate dsDNA and ssDNA¹² per vaccine dose (Deisher data, submitted for publication attached "Environmental Triggers and AD submitted manuscript").

While the case in question here involves MMR II only, our epidemiological work has connected each stage of the autism epidemic to the introduction of each of these vaccines. These contaminants, not the vaccine itself have been the focus of our work, thus the identity of the vaccine is relevant only due to the load of contaminants in it, i.e. the dose. The dose of contaminants given to a child, appears to have a profound effect. This is consistent with the conclusions of numerous scientists now engaged in research attempting to develop useful therapies through genetic engineering. Indeed, the change in the upward slope of the line that traces the increasing epidemic of autistic disorder in the US (graph below) is greatest after introduction of the more heavily contaminated Varivax (chickenpox) vaccine, suggesting a dose relationship between the human fetal DNA contaminants and autism disorder.

¹² Single stranded DNA.

**Human Fetal DNA and Retrovirus contaminants in Vaccines
Coincide with Autism Changepoints**



HERVK

Human endogenous retrovirus K (HERVK), a contaminant of the Varivax and MMRII vaccines (20) is a retrovirus that integrated into human germline cells relatively recently in human evolution and is inherited in a Mendelian fashion as an endogenous retrovirus¹³. Such retroviruses are generally inactive. Thus, experts have considered the presence of endogenous retroviruses in the human genome to be innocuous. However recent evidence has shown that HERVK can be reactivated (24) (25) (26) (27) or even maintain its activity in present day humans (28) and integrase activity from homologous HERVK sequences has been reported (25). Active HERVK integrates preferentially in transcription units,¹⁴ in gene-rich regions¹⁵, and near features associated with active transcription units and associated regulatory regions (29).

Recent evidence has shown that HERV transcripts are elevated in the brains of patients with schizophrenia or bipolar disorder (30) (31) and in the peripheral blood mononuclear leucocytes¹⁶ of patients with autism spectrum disorders (32). This retrovirus has also been associated with several autoimmune diseases (33) (multiple sclerosis) (34) (rheumatoid arthritis) (35) (schizophrenia) HERVK is in the same family of retroviruses as the MMLV (36) virus used in the gene therapy trials referenced first above and later in this paper, in which inappropriate gene insertion led to subsequent additional somatic mutations and cancer in 4 of 12 young boys in a study, i.e. 40% (2).

The paper by Victoria et.al. (20) reports the presence of fragments of the HERVK genes in Varivax and MMRII by RT-PCR, yet the portion of the HERVK gene present was not determined. Our epidemiological study showing a correlation between the vaccines in issue and neurological malfunction is scientifically strong evidence that the HERVK gene fragment present in vaccines more likely than not codes for the integrase or the envelope protein, thus is active and induces gene insertion (25) or neuroinflammation (37) (38). This presents a highly probable danger to recipients of these vaccines. As with all other strong evidence of gross errors in the assumptions underlying the licensing of the use of human cell lines for the propagation of vaccine viruses published since 1999, the FDA has not followed up on the 2010 Victoria publication with the post market safety review made possible by the existence of the Vaccine Safety Data Link

MECHANISM OF DNA INSERTION

Mammalian cells can take up extracellular DNA fragments by receptor mediated endocytosis¹⁷. Uptake is most efficient at low concentrations of extracellular DNA (39) and peaks 2 hours after addition of the DNA fragments to cell culture (40). In the extracellular concentration range of 0.1

¹³ An 'endogenous' retrovirus is the result of retroviral integration in germline cells which causes the organism, i.e. the person, to carry the retrovirus as an integral part of their genome. Thus, it can be carried from generation to generation.

¹⁴ A stretch of DNA being transcribed into an RNA molecule. RNA molecules are single stranded nucleic acids composed of nucleotides. RNA plays a major role in protein synthesis as it is involved in the transcription, decoding, and translation of the genetic code to produce proteins. Nucleotides are biological molecules that form the building blocks of nucleic acids (DNA and RNA).

¹⁵ Regions of the genome containing many protein coding genes., Sumner AT, de la Torre J, Stuppia L (August 1993). "The distribution of genes on chromosomes: a cytological approach". *J. Mol. Evol.* **37** (2): 117-22. doi:10.1007/BF02407346. PMID 8411200

¹⁶ Mononuclear leukocytes, are white blood cells with a one-lobed nucleus.

¹⁷ is a process by which cells internalize molecules (endocytosis). The routes that lead inward from the cell surface to lysosomes start with the process of endocytosis, by which cells take up macromolecules. In this process, the material to be ingested is progressively enclosed by a small portion of cell membrane containing proteins with

to 7 μM ,¹⁸ oligonucleotides (small bits of nucleic acids) readily enter cultured cells through receptor mediated uptake (41) (42) (43) (44), reaching intracellular and nuclear (41) (45) (46) (47) concentrations which equal or exceed that of the extracellular medium within 2-4 hours (48)¹⁹. Empirical experiments have shown that addition of placental DNA fragments of 500 base pairs in length contributed approximately 4% of a cell's genomic content per hour of incubation – roughly 40-50% of fragmented DNA added to cell culture will be taken up by a cell and 10-20% of the added DNA will be delivered to the nucleus, demonstrating the rapidity with which DNA can enter a cell (39) (page 3 column 2) (49) (page 4 of 9).

Current recommendations are that the level of residual cell-substrate DNA should be less than 10 ng (10^{-10} ; ng is a nanogram, one billionth of a gram) per dose with a median DNA size of 200 bp or lower. Assuming the residual cell substrate DNA in the Varivax has been fragmented according to FDA guidance and is approximately 215 base pairs in length as it is in the Meruvax vaccine, and is all double stranded, then each Varivax vaccine administers 15.2 picomoles (15.2×10^{-12}) of contaminating DNA. A one-year old child has approximately 800 mls of blood and 1.6Ls of extracellular fluid volume, and a two year old child has approximately 1L of blood volume and 2Ls of extracellular fluid volume (3L total), and approximately 100 trillion cells. The extracellular fluid volume around each individual cell is then approximately 24-30 femtoliters (0.02 trillionths of a liter) of fluid and the relative concentration of DNA seen by a cell at the site of injection could be estimated to be between 5 to 7 μM (if the DNA is instantaneously distributed evenly through the entire fluid volume of the child's body) and 500 to 700M (if the DNA is concentrated at the extracellular space of a single cell), a concentration range clearly containing the range of 0.1 to 7 micromolar for ready cell uptake (0.1 to 7 μM).

The potential for exogenous DNA to enter the nucleus of a cell and insert into the genome of that cell is a well-established biologic process. Indeed, nuclear transfer of mitochondrial DNA fragments (NUMTs) is an ongoing process in mammals, including humans, today (50).

Insertional mutagenesis refers to the alteration of the cellular genome by the integration of viral or recombinant DNA, and is the default outcome of transgene integration, unless homologous recombination is achieved (51) (page 1 Introduction column 2 lines 4-7; this review also demonstrates how inaccurate expert opinions are in predicting actual insertional mutagenesis events). Theoretically based estimates from several decades ago put the probability of exogenous DNA integration causing insertional mutagenesis/oncogenesis at 10^{-16} or less (52) (53) (14), and therefore, the risk was deemed negligible although experts continued to meet and debate the dangers without resolution or significant study (7) (page 7 lines 15-21, page 29 lines 10-22, page 63 line 22 – page 64 line 6.) One WHO sponsored meeting concluded that "Additional calculations suggest that the risk of insertional mutagenesis that could lead to a neoplastic event is extremely small. In one recent report, it was predicted that a 10-microgram dose of DNA would result in the inactivation of two independent tumor-suppressor genes, by insertional mutagenesis, within a single cell of a vaccine recipient in only one of 10^7 recipients. These very low calculated levels of risk are consistent with the limited human and animal experience to date" (9) (page 32 lines 16-23). Similarly, the risk of insertional mutagenesis/oncogenesis (the production or causation of tumors) from gene therapy using retroviral fragments and short DNA segments was also deemed, intuitively, to be negligible if not nil (54) (page 3 column 2 paragraph 2) (55) (page 1 column 2 paragraph 3) (1) (page 1 column 2 lines 4-5) (56), (51) (page 1 Introduction column 2 lines 9-11) ^{20 21 22}. As with all theoretical conclusions about these processes, clinical trials have demonstrated empirically that the risk of insertional oncogenesis was dramatically higher than experts had predicted (57). As mentioned above, 4 of 9 or 10 boys developed cancer in one clinical trial (1). 40% is much higher than the predicted number of 10^{-16} or less (52) (53). Unfortunately, the focus of concerns amongst scientists in academia, in industry and at the FDA has been on the potential of residual DNA for oncogenicity or infectivity, not on the potential for the induction of subsequent gene mutations following genomic insertion of DNA fragments, although this danger was indeed discussed during the 1999 FDA workshop entitled "Evolving Scientific And Regulatory Perspectives On Cell Substrates For Vaccine Development" (7) (pages 61-75.) Then the

receptor sites specific to the molecules being internalized. The Amgen web cite plays a video showing a simplified cartoon representation of this process which I may play at any hearing.

¹⁸ Micromolar, 10^{-6} mol/dm refers to the concentration of a substance in a solution.

¹⁹ Again, experimentation on humans is not morally acceptable, thus to determine if there is an effect on humans science uses human cell lines, similar to those used in the manufacture of the vaccines here in issue.

²⁰ Retrovirus vector insertions were not at all randomly distributed, and had far more influence on the biological fate of engrafted cells than had initially been anticipated.

²¹ We interpret these findings as the consequence of an insertional mutagenesis event, a risk ...that has previously been considered to be very low in humans. (54)

²² Previous studies in animals predicted that retrovirus-mediated gene transfer poses a potential, but remote, risk of insertional oncogenesis. Until this report, retroviral insertion in the context of gene therapy has been considered an untargeted and largely random event. (55)

²³ Before 2002, the likelihood of cancer induction by insertional mutagenesis had mostly been considered negligible.

FDA preferred to do 'intuitive' rather than 'experimental' analysis (7) (page 87 lines 10-15) ²⁴, and despite the empirical data now available from the human gene therapy work including the abortive trials demonstrating that cancer was induced due to inappropriate viral fragment and DNA fragment insertion that led to subsequent additional somatic mutations (2) (3), establishing that prior estimates were grossly wrong, no action has been taken to re-examine the significance of these revelations to vaccine safety. Again the respondent has refused to use the Vaccine Safety Data Link to see if these vaccines are associated with the type of adverse effect found to be likely by all the work done by science for the last 14 years on genetic engineering.

While ignoring a post marketing study, due to concern about the potential for a complete oncogene to be carried through the manufacturing process and inserted into the recipient's genome, the WHO and FDA recently issued a recommendation calling for DNase treatments to make the residual DNA shorter than the length of a gene to lessen the risk of oncogenicity (12)(page 41 section C2) (58) (page 7 of 9 column 1 lines 1-4 and 44-50). Unfortunately, it appears that in an effort to provide safety these regulatory agencies may have recommended the worst possible solution, since short DNA fragments are known to integrate into the genome in a species specific manner leading to mutagenesis and/or genomic instability. Perplexingly, it is documented that the FDA was aware at the time of some of these recommendations that DNA fragments could become even more dangerous through the induction of the chromosomal breaks or rearrangements which result from DNase treatment (8) (page 9) (7) (page 76, 151-154 and 256).

As measured by our institution, the residuals in the rubella portion of the MMRII vaccine are approximately 215 base pairs in length (attached "Environmental Triggers and AD submitted manuscript"). While Merck reveals that "*The nearly 2 ug of unmodified mammalian DNA present in each dose of Varivax™ exceeds that present in any other approved childhood vaccine*" they do not disclose the fragment size of the contaminating human fetal DNA.

In the Varivax Summary Basis for Approval, Merck also acknowledges that 5% of the human fetal MRC5 cells used to manufacture the vaccine carry a clonal 7:12 chromosomal translocation (13) (page 3 lines 12-16). While Merck tested the tumorigenicity of this anomaly in nude mice, they do not disclose or they did not perform any experiments to determine the efficiency of DNA insertion into human cell lines, but relied on "supplemental expert testimony," to conclude that the high levels of contaminating DNA and the presence of a chromosomal translocation "did not pose a safety risk" (13) (page 3 lines 21-26). Again, manufacturing companies as well as the FDA prefer 'theory' and 'expert opinion' over actually conducting safety studies to define the risks. The only empirical work was that stated above, nude mice and non-human primate studies. This was despite the lessons learned from the unfortunate human gene therapy clinical trials, that demonstrated the inability of animal studies to predict insertional mutagenesis/oncogenesis risk in humans (FN 4) (56) (page 1 column 3 lines 35-40 page 2 column 1 lines 1-3), (59), documenting the necessity of evaluating human DNA genomic incorporation in human cell lines rather than animals. Furthermore, when insertional mutagenesis is apparent in animals, young animals appear to be more sensitive to the mutagenesis than adult animals (60) (61).

AGE AS A FACTOR IN SUSCEPTIBILITY

During the period from birth out to three or more years, human brain development is an active process, with neural circuits being established, pruning of unused dendritic synapses going on, and nerve cell death occurring on a massive scale (62) (63). During periods of intense brain cell death such as this, DNA not otherwise found extracellularly would be present and serve as the target for autoimmune attacks, originally triggered by exposure of a young child to the fetal DNA fragments found in vaccines.

Of course we cannot experiment on human children. We must use animals (mammals) as surrogates and science has observed cells dying within the cortex, the hippocampus and the olfactory bulb within a 5 to 25-day period, commencing about day 5 post-natal (64). In all mammalian species examined, postnatal brain cell death ranges between 30-70% of neuronal content (65). In a human child the rat nerve cell death period would correspond to between birth and 3-5 years of age, the time period when children are vaccinated with and exposed to these potentially auto-immune eliciting and/or invasive, fetal DNA contaminants.

²⁴ DR. KRAUSE: You are raising a very legitimate point. To my way of thinking, these are the kinds of questions which need to be answered. The question is do they need to be answered on an intuitive basis or do they need to be answered on an experimental basis. (2)

In the mammalian genome, double strand DNA breaks (“DSBs”) occur during cellular processes such as DNA repair, recombination and replication; the early prophase of meiosis²⁵, V(D)J recombination²⁶ or as a result of exposure to DNA damaging agents. Repair of these DSBs is necessary to maintain genomic stability. This repair process provides the opportunity for insertion of DNA fragments, in addition to the phenomenon of illegitimate recombination²⁷. Mammalian cells can repair DSBs both by homologous recombination²⁸ and by illegitimate recombination of DNA fragments (IR). However, homologous recombination frequencies are 100-1000 fold less than illegitimate recombination.

This fact is the major obstacle to gene targeting in mammalian cells. Compared to homologous recombination, illegitimate recombination does not require any extended sequence homology and is more common than homologous integration in most mammalian cells (66) (67). It has been demonstrated in vitro (i.e. in cell culture in the laboratory) that illegitimate recombination in mammalian cells could cause mutations and genomic structural rearrangements associated with tumors (68). In contrast, in yeast, homologous recombination is much more frequent than illegitimate recombination, which is rare. (67). Therefore, opinions and statements about integration efficiencies need to be carefully and clearly made distinguishing which species is referred to and what type of integration event is being described. In mammalian cells, illegitimate integration, the primary concern associated with vaccine contaminants, is a much more common event than homologous recombination (69) (70) (71) (72).

Indeed, at the 2011 IMFAR meeting, presentations demonstrated that genes involved in DSB were differentially expressed in ASD (Courchesne E, oral presentation and Outwin 2011 Ref 73b). Faulty DSB is known to be involved in many diseases (73). DSBs occur both in somatic and germ line cells, and can be programmed, such as in somatic cells for immunoglobulin hypermutation and class switching, or the result of DNA replication, or spontaneous DNA hydrolysis or cellular metabolism (74) (75). Toxins and chemotherapeutics can be inducers of DSBs in somatic cells. In the case of various lymphomas, we know that the addition of a toxin or chemotherapeutic induced DSB on top of a programmed class switching DSB leads to cancer (75). In summary, this research reveals that the genetic susceptibility of some children to the development of ASD is due to the genes involved in DSB being differentially expressed (i.e. not normal). Together with the presence of recombination hotspots in genes that have been associated with ASD (discussed below), these differentially expressed DSB genes constitute an underlying predisposition to development of ASD as a result of insertions of fetal DNA. Thus, children with this genetic condition (abnormal DSBs) are extremely susceptible to such insertions.

Meiotic recombination (MR) involves highly regulated pathways of double strand break formation and repair. MR occurs at clustered sites within the human genome, termed recombination hotspots, the vast majority of which are located outside of genic regions (76), presumably to reduce the potential for lethal results after MR. The locations of meiotic recombination hotspots in the reference human genome were recently mapped (77), and the zinc-finger DNA binding protein, PRDM9, has been demonstrated to bind to a degenerate 13-mer DNA motif (CCNCCNTNCCNC) found at specific locations in the genome which are associated with a large percentage of these (78) (79). Allelic homologous recombination (HR) during meiosis drives population diversity, and, beyond meiosis, HR has also been shown to increase diversification of immunoglobulin genes in somatic cells via the Rag proteins (80), and to repair genomic damage in somatic cells. Recent studies have demonstrated that HR can also play a critical role in genetic disorders (81); colocalization of mapped meiotic hotspots and disease-causing chromosomal translocations have been demonstrated in mouse somatic cells (82). Interestingly, the presence of the 13-mer motif has been found to be associated, not only with meiotic recombination events, but with the common mitochondrial deletion and with the disease ichthyosis (79). Myers et al. have found over 25,000 recombination hotspots in the human (77). Internal work at SCPI has identified over 280,000 occurrences of the 13-mer motif in the human genome.

Sound Choice Pharmaceutical Institute (SCPI) has conducted research into the overlap of genomic MR hotspots, degenerate 13-mer PRDM9 DNA binding motifs and known disease associated genes, focusing on genes published to be associated with autism (autism associated genes AAGs). On the human X chromosome, the presence of MR hotspots and 13-mer motifs are enriched in genes associated with ASD (Table I). Interestingly, sites of HR (double strand breaks) have been demonstrated to be further susceptible to additional DSBs and mutations (82) (and Holbeck Ref 82b) (83).

²⁵ The first stage of a special type of nuclear division which segregates one copy of each homologous chromosome into each new cell that fuses with another during fertilization. Mitosis then maintains the cell's original number of chromosomes (for example, one diploid 2n cell producing two diploid 2n cells; one haploid n cell producing two haploid n cells; etc.). This is generally restricted to the gonads.

²⁶ is a mechanism of genetic recombination in the early stages of immunoglobulin (Ig) and T cell receptors (TCR) production of the immune system.

²⁷ Illegitimate recombination (IR) is the process by which two DNA molecules not sharing homology to each other are joined.

²⁸ a type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical molecules of DNA. It is most widely used by cells to accurately repair DSBs.

Table I: MR Hotspots containing 13-mer PRDM9 binding motifs are enriched in disease associated genes on the X chromosome.

X-Chr	# genes	# genes containing 13 mers	# 13 mers within genes	#13 mers per gene	# genes containing MR hotspots	# MR hotspots within genes	# MR hotspots per gene	# genes with 13mer within a MR hotspot	# 13 mers within MR hotspots	#13 mers within MR hotspot per gene
genes	1002	656	4505	4.50	141	337	2.39	112	2242	2.24
autism associated genes	38	36	546	14.37	17	89	5.24	16	410	10.79
genes		65%			14%			11%		
autism associated genes		95%			45%			42%		

Table I legend: Lists of disease-associated genes were downloaded from the ACGMAP website (Autism Candidate Gene Map Database, 2011) and the AutDB website (Autism Database, 2011). Coordinates for these genes were downloaded from the UCSC Human Genome Browser. Hotspot locations downloaded from (77) from build 34 were updated to human genome build 37 with the LiftOver tool from the UCSC browser. An overlaying software was written to automate the overlay of hotspot and autism gene locations and to find any hotspots contained wholly or partially within genes.

Over 350 genes have been associated with ASD. Genomic anomalies include common genetic variations (84), changes in chromosomal structure (85), and rare mutations (86). Recently, de novo deletions and duplications have been identified in up to 10% of simplex ASD, indicating environmental influences on the genetics of ASD (87) (88). 10% may well under-represent de novo mutations as methods are limited to detecting large de novo CNVs and do not fully capture smaller mutations (89). Furthermore, each specific mutation is found in only a very small percent of cases, highlighting the complexity of genomic impacts on ASD and the challenge of understanding the de novo mutation process. Network mapping is revealing downstream links between these diverse genomic mutations and ASD phenotype (90), yet we do not understand the process by which diverse genomic sites are targeted for mutation. Altered double strand break formation and repair pathways (DSB) may be a commonality among the extremely diverse genetic mutations observed in ASD.

Studies within the past decade have clearly established the rate of in vivo genomic integration of large size plasmids²⁹ at 5×10^{-5} . Ledwith (91) injected plasmid DNA (>4.9 kB between 100 and 300 micrograms corresponding to 24-30 trillion plasmids, and in comparison Varivax probably contains 9.2 trillion fragments) intramuscularly to mice and showed that integration rate measured by PCR³⁰ after gel separation of plasmid from genomic DNA was 1-8 per 150,000 cells between 6 to 26 weeks after injection (0.005% efficiency). Manam et al (92) injected between 100 and 160 micrograms DNA intramuscularly to mice (between 5.1 and 6.6 Kb lengths corresponding to 15-19 trillion plasmids) or 1.1 to 1.4 milligrams (between about 150 and 300 trillion plasmids) intramuscularly to guinea pigs and six weeks later detected < 1-8 copies per 150,000 cells using the same methods as Ledwith et. al. While the integration rates of these large genes and plasmids are low, they are clearly not 'nil'. Moreover, gene therapy studies have shown that supercoiled DNA < 1 kilobases long is resistant to DNase digestion and can be transported to the nuclear region more readily than longer DNA (93) and Lechardeur (95).

Furthermore, Manam (92) table 5) found persistent long term expression of the genomic integrated DNA in injection site muscle and skin biopsies and in draining lymph nodes 6 weeks after intramuscular injection, a long term persistent genomic incorporation which has been confirmed in other in vivo DNA vector injection experiments (Riede 2015 ref 92b figure 1 C and D). Discussions in the published papers about the dangers of this long term genomic incorporation have created an imaginary field of data by citing to other publications that cite to a single publication (Riede 2015 page 632 left column and (92) page 278 last sentence to page 279 first sentence and (91) page 271 right column) regarding spontaneous human mutation rates (Cole 1994 ref 92c), giving the appearance that multiple publications rather than the single 1994 publication provides spontaneous human mutation rates. While spontaneous disease associated human mutation rates in some cell types are indeed 3 orders of magnitude larger than the genomic insertion rates measured with exogenous DNA plasmids or fragments, the authors fail to discuss the fact that spontaneous mutation rates vary among cell types and are equivalent to the genomic insertion rates of exogenous DNA in some cases, making

²⁹ a small DNA molecule that is physically separate from, and can replicate independently of, chromosomal DNA within a cell.

³⁰ Polymerase Chain Reaction (PCR) is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Polymerase Chain Reaction (PCR)

the genomic insertion a more dangerous artifact than the authors would like to concede (Cole 1994 ref 92c) and Morley AA 1996 ref 91b and Holmquist ref 133.

In contrast to the integration of large DNA gene lengths, integration of short DNA fragments has been shown to be much more efficient. Integration is maximal when fragments are between 100 and 1000 base pairs in length Ref 94b Tsuchiya 2005 Figure 2B (3-6 trillion fragments) (95) (page 5 figure 2) and ref 95b Lukacs 2000. In vitro small fragment homologous replacement (SFHR) has demonstrated an insertion efficiency between 0.1-10% for DNA fragments ranging in size from 400 to 800 base pairs (96) (97) (98). McNeer et. al. have investigated the efficiency of small DNA fragment integration following nanoparticle versus nucleofection transfection³¹. McNeer et. al. utilized synthetically produced 50 base pair nucleotides targeted to the beta-globin gene exon 2 intron 2 transfected into human CD34+ stem cells. They measured between 0.05% and 0.9% integration/mutation when they delivered $0.24-1.2 \times 10^8$ ssDNA particles using either limiting dilution or PCR analysis (99). Schleifman, using similar transfection methods as McNeer, demonstrated up to a 2.46% in vitro THP-1 and K562 human cell lines homologous recombination efficiency which persisted out to 3 months, which was about 10-90 fold more efficient than DNA alone, putting DNA only electroporation incorporation efficiency at about 0.03-0.25% (100). Other publications demonstrate genomic integration efficiencies between 0.4 to 4% with 3 to 9 trillion ssDNA fragments between 390 and 606 nucleotides in length (Ref 100b Colosimo 2000, and Ref 94b Tsuchiya 2005).

As another example, Bedayat, et. al. determined the insertional genomic frequency in human B lymphocytes carrying a single base pair substitution (HPRT1 cells), and quantified efficiencies between 0.1 and 2% for 1-10 million DNA fragments per cell between 579 base pairs in length, aided by electroporation³² (96). Another group, Knauert et. al., documented genomic integration efficiencies for synthetically produced ssDNA fragments between 30 and 69 base pairs in length of approximately 0.01% in CHO cell lines³³ (102). While Bedayat, Sangiuolo, Goncz and Knauert (referenced above) used small homologous DNA fragments and electroporation, nucleofection or micro-injection to enhance cellular delivery of the DNA fragments, other investigators have successfully integrated DNA fragments derived from digestion³⁴ of entire mammalian genomes and did not require electroporation or other enhanced delivery methods.

Indeed, efforts to develop gene therapy have documented that short DNA fragments can and do insert into a recipient's genome with an efficiency of up to 20%, a number that is dramatically larger than nil. Yakubov et. al. (2007) utilized human placental DNA fragments of 200 to 3000 base pairs in length and demonstrated spontaneous uptake and insertion up to 4% of the genome of MCF-7 human cell line, and also demonstrated the species specific nature of this genomic incorporation because fragments of salmon sperm DNA did not integrate into the human MCF-7 cells (49). Similarly, Sound Choice Pharmaceutical Institute has demonstrated genomic incorporation rates of 0.2 to 0.6% of recipient genome 24 to 49 hours after addition of DNA fragments of approximately 350 base pairs in length to the culture media for U937³⁵ or NCCIT³⁶ cells. DNA fragment uptake was spontaneous and did not require cell permeabilization or transfection (presented at the International Meeting For Autism Research (IMFAR 2012) and poster attached IMFAR_2012_DNA uptake_poster KK_050312). Colosimo also demonstrated in vitro gene integration of small homologous fragments in 1-10% of CFTR mutant human epithelial cells and 2.4% integration efficiency in B-thalassemia erythroid cells (104) (105).

Most significantly, small fragments of DNA have also been shown to integrate into the genome efficiently in vivo. Jensen et. al. have successfully delivered and integrated small DNA fragments to the mouse liver using tail vein injections for administration (103). McNeer et al delivered approximately 300 trillion DNA fragments up to 60 base pairs in length to mice by tail vein injection, and demonstrated 0.01 to 0.04% genomic integration with DNA alone that was increased to up to 0.12% with triplex forming PNA³⁷ (fig 8e). Genomic integration of DNA without PNA was highest in the cells found in the bone marrow, liver and thymus (106). Furthermore, using these same techniques, McNeer and Schleifman have also demonstrated in vivo targeting efficiencies, using systemic intravenous injection, of between 0.05% (page 5 of 12) and 0.43% (page 4 of 12) using deep gene sequencing of bone marrow and spleen cells respectively (106). This study also demonstrated the species specific nature of

³¹ **Nucleofection** refers to electroporation.

³² a significant increase in the [electrical conductivity](#) and permeability of the [cell plasma membrane](#) caused by an externally applied [electrical field](#)

³³ **Chinese hamster ovary (CHO) cells**

³⁴ Describing methods used to fragment the human genome into DNA fragments similar in size and composition to those found in the vaccines.

³⁵ A human cell line consisting of Lymphoblasts from lung, immature cells which typically [differentiate](#) to form mature [lymphocytes](#)

³⁶ A human cell line established by Shinichi Teshima (National Cancer Institute, Tokyo, Japan) in 1985 from a mediastinal mixed germ cell tumor.

³⁷ Triplex-forming peptide nucleic acids (PNAs) are powerful gene therapy agents that can enhance recombination of short donor DNAs with genomic DNA, leading to targeted and specific correction of disease-causing genetic mutations.

DNA integration in that mice chimeric³⁸ for human CD34+³⁹ stem cells containing a CCR5 mutation⁴⁰ showed uptake of the injected fragment only in the human CD34+ stem cells but not in the mouse CD34+ stem cells in these chimeric animals (page 3 and 4 of 12).

Efficient uptake of short DNA fragments into stem cells was further demonstrated by transfer experiments in which lethally irradiated mice were rescued by injection of cells from chimeric mice that had integrated a CCR5 short DNA fragment. The rescued mice also carried the CCR5 short DNA fragment insertion, demonstrating that insertion into stem cells was efficient and permanent (106). Such high in vivo efficiencies in the spleen and bone marrow may indicate a preference for uptake and integration of the ssDNA fragments into stem cells, rather than fully differentiated, non-dividing somatic cells. The efficiency of DNA integration into stem cells has also been demonstrated by other investigators. Chin et. al. have observed measurable efficiency of small fragment DNA only integration in primary human CD34+ stem cells (107) (figure 4), and Liu showed an efficiency of small DNA fragments overall integration of 1.7% to human CD34+ stem cells in vitro (108).

Observations that stem cells appear to be a high efficiency target for small fragment DNA incorporation are promising for those pursuing gene correction therapies, yet ominous as regards concerns for residual DNA in biologics. Preferential incorporation into stem cells provides a route for widespread dissemination of any mutation throughout the body, including the brain and a continued long term presence of any disruptive mutation as stem cells are long lived, self-replicating cells of the body.

Furthermore, the efficiency of DNA integration has been demonstrated to be enhanced by agents which interfere with lysosomes⁴¹. As mentioned previously, Chin et. al. demonstrated 0.05% integration (fig 2b) efficiency by homologous recombination in Chinese Hamster Ovary (CHO) cells with DNA alone, and excellent efficiency in primary human CD34+ stem cells (107). Efficiency was greatly enhanced by the lysototropic agent chloroquine (from 0.04 to 0.16% fig 3a), which interferes with lysosomes and protects DNA from degradation. Lysototropic agents include chloroquine, NH₄Cl, MeNH₂, alkylamines, and monesin, which can inhibit fluorescence dequenching observed at pH 7.4 by reducing the pH in the lysosomes which reduces their ability to deal with invasive DNA. Stabilization of DNA can be enhanced as well by Ethylenediaminetetraacetic acid ("EDTA") and sodium nitride ("NaN₃") (109) (110) (111). The Varivax vaccine contains "EDTA" in the final product, yet no studies on the stabilizing nature of EDTA for the DNA fragments contained in the Varivax vaccine have ever been conducted (attached "Varivax Summary for Basis of Approval", Varivax package insert page 1 Description section line 14).

Numerous studies have established the ability of species specific DNA to accumulate intracellularly and insert into a host's genome at an appreciable rate. Additional questions thus arise regarding how such an insertion would impact neural development or would occur within a cell in the central nervous system, as autism spectrum is a neurodevelopmental disorder. Several plausible mechanisms exist, with large bodies of supporting scientific literature. However, at this point we do not know the exact mechanism for how DNA insertion reaches the brain to elicit autism spectrum disorder onset. Published studies in mice (112) (an autoimmune model of autism in which bone marrow stem cells play a role) (113) (describes in vivo viral induced DNA breakage in bone marrow cells elicited by viruses such as measles and rubella (114) (describes a two hit model highlighting the importance of the thalamus, where underlying genetic susceptibility requires a second somatic mutation to develop Tuberous Sclerosis, a condition in which 25-50% also have autism) (115) (describes a mouse model of mosaic genetic mutation of neural progenitor cells inducing tuberous sclerosis and resulting mitochondrial and oxidative abnormalities) (116) (describes a model where abnormal DNA methylation in response to a mutation leads to Rett Syndrome and autism), anecdotal evidence, publications and oral presentations from autism spectrum experts provide support for two distinct potential mechanisms, and it is quite possible that delivery of the fetal contaminant insult varies among children, with some children affected by de novo mutations to the stem cell common to microglial cells and hematopoietic lineage cells (email communications Laurette Janak) but other children harboring brain specific mosaic mutations (Courchesne E, International Meeting For Autism Research, 2011 oral presentation and). Ref 117b Bushman 2013.

Both of these delivery routes are furthermore amenable to ultimate disease induction due either to gene mutations and protein dysfunction or to autoimmune attacks as the ultimate culprit. Genomic insertion into a blood (hematopoietic) stem cell would generate somatic microglial cells with altered function, and as the microglial cell is a critical immune modulating cell in the central nervous system, disrupted functionality through

³⁸ a single [organism](#) (usually an [animal](#)) that is composed of two or more different populations of genetically distinct [cells](#) that originated from different [zygotes](#) involved in [sexual reproduction](#)

³⁹ The CD34+ is a cell line of Progenitor Cells which contain two main cellular subpopulations, hematopoietic and endothelial progenitor cells

⁴⁰ Cysteine-cysteine chemokine receptor 5 (CCR5) is found in the cell membranes of many types of mammalian cells, including nerve cells and white blood cells. The role of CCR5 is to allow entry of chemokines into the cell³—chemokines are involved in signaling the body's inflammation response to injuries. The mutation renders the receptor inoperable.

⁴¹ **Lysosomes** are cellular [organelles](#) that contain acid [hydrolase](#) enzymes that break down waste materials and cellular debris, including stray DNA fragments etc.

insertion and mutation in this cell would manifest itself as an apparent disease with immune dysregulation involvement. Transport of the fetal DNA fragments and retroviral fragments contained in the vaccines through the axonal retrograde transport system could result in DNA insertions and mutations in the central nervous system brain cells themselves. The result of DNA insertions and mutations could elicit disease based on expression of aberrant proteins or to autoimmune attack because of the expression of aberrant proteins by the mutated cells.

Also, fundamentally, exposure of a child to fragments of human fetal (primitive) non-self DNA could generate an immune response that would cross-react with the child's own DNA, since the contaminating DNA could have sections of overlap closely similar to the child's own DNA. Lupus is one example of an auto-immune disease where the immune attack is targeted towards the person's own DNA. Because of this danger, Merck conducted studies to "To assess whether these impurities could induce a harmful anti-DNA autoimmune response, serum IgG anti-DNA antibody levels were monitored in a cohort of 293 subjects who were immunized and boosted with V ARIV AX®. A comparison of anti-DNA titers before immunization and at 6 weeks and 1 year after boost showed no significant change in either the average anti-DNA antibody titer or the frequency of elevated anti-DNA titers in immunized subjects." (13) (page 13). Unfortunately, these anti-DNA autoimmunity safety studies were conducted in the early 1990s, and during those years children received just two shots containing residual human fetal DNA and retroviral fragments; the MMRII and the Varivax. Since 2005 Hepatitis A containing vaccines have added additional human fetal contaminants to children at around 12 months of age and since 2008 a significant percentage of young children have received human fetal DNA contaminants as young as 2, 4 and 6 months of age from the Pentacel vaccine. With the current ACIP and statewide vaccination recommendations, children may be exposed to as many as 7 or more fetal DNA contaminated vaccines before they are 2-3 years old. While Merck acknowledged the relevance of testing for this response prior to the 1995 approval of Varivax, we cannot find any evidence that additional testing for auto-immune responses has been done as more fetal DNA contaminated vaccines were introduced to the vaccination schedule, a fact which makes the dangers of anti-DNA autoimmunity greatly amplified. Furthermore, antibody measurements in the serum of autistic versus healthy age and sex matched controls born between 2002 and 2009 demonstrates anti-neuronal, anti-dsDNA and anti-ANA antibodies with 90% of girls and 53.3% of boys showing positive autoimmune (References 18b,c,d and e Al-Ayadhi 2012 and Mostafa 2012, 2014, and 2015).

Empirical data from studies of leukemia and autism in children with Down Syndrome provide further evidence that residual fetal DNA fragments insert into blood stem cells, causing subsequent mutations that lead both to leukemia and to autism. Children with Down Syndrome have been demonstrated to have reduced ability to repair DNA damage, particularly DNA damage to stem cells. (ref 118a Morawiec 2008; Ref 118b Zana 2006) This renders them susceptible to agents that would cause double strand breaks or alter the genome in any way. Not only does the fetal DNA present the potential to insert into the child's genome, foreign DNA is also a known method (one of a very few established methods) to induce double strand breaks and DNA damage (53) (table 3 page 49). In regards to the alternative mechanism of how DNA insertions might impact the brain –the potential for direct induced mutations in brain cells - several studies and oral presentations have described observed de novo mosaic mutations among nerve cells, although this phenomenon is currently grossly understudied (Courchesne E, International Meeting For Autism Research, 2011 oral presentation and (119) and ref 117 Bushman 2013)). Recently, Michaelson et. al. have published that genes expressed in the brain and additionally that genes associated with ASD are more hypermutable than other genes (120) (page 7 right column lines 1-10). Additionally, Sound Choice Pharmaceutical Institute has presented several posters on the concentration of hotspots for DNA break induction in genes associated with ASD (IMFAR 2011 and 2012), and the presence of these hotspots has been demonstrated to be a predisposition for somatic disease inducing mutations (79) page 3 figure 3 and page 4 left column lines 4-6 and right column lines 8-10 (81) reviewed in introduction (121) figure 10. . Therefore, brain cells and genes associated with ASD, like primitive stem cells, appear to have an underlying predisposition making them susceptible to agents that would damage or mutate cellular DNA.

In the preceding paragraph I introduced the concept of 'hotspots'. Hotspots are sites in the genome where exchange of chromosomal material between the maternal and the paternal chromosome occur most readily during meiosis (germ cell generation for reproduction); this is called meiotic recombination. This process creates genetic diversity in our offspring, and is beneficial in that sense. However, sites in the genome where double strand breaks have occurred, such as sites of meiotic recombination, have been shown to be susceptible to subsequent double strand breaks and disease causing mutations have been documented in these areas (79) (81) reviewed in introduction (121) . . Similar to the susceptibility of stem cells to take up and integrate foreign human DNA into their genomes, an underlying susceptibility for mutations has been identified among those genes that have been shown to be associated with ASD (120)]. Double strand breaks (DSBs) can lead to chromosomal instability and improperly repaired DSBs can lead to human disease. Diseases known to be influenced by genomic deletions or insertions include cancer (76), hereditary neuropathy [(76), mitochondrial syndromes (77)], ichthyosis (77) (121), Nijmegen breakage syndrome (NBS) (122), autism spectrum disorder (123) (124), schizophrenia (125) and others.

To briefly review : faulty DSB repair is known to be involved in many diseases, and normal cell biology is associated with DNA breaks in several ways. Double strand breaks (DSBs) occur both in somatic and germ line cells, and are programmed in germ line cells for meiotic recombination and in somatic B cells for immunoglobulin hypermutation and class switching. Additionally, it is estimated that there are ~10 DSBs each time the mammalian genome is replicated (74), and thousands of single-strand DNA breaks occur in each somatic cell each day, and when a replication fork encounters a single-strand break, the fork collapses and a DSB is the result. Toxins and chemotherapeutics are also known inducers of DSBs in somatic cells (80). DSBs can be repaired by various pathways, including homologous recombination (HR), non-homologous end-joining (NHEJ) or single strand annealing (SSA), and each repair pathway is most likely a stochastic competition regulated by the availability of protein machinery needed for the repair during a particular part of the cell cycle. HR and SSA are typically used more often during the S and G2 part of the cell cycle, while NHEJ can be used for most of the cell cycle [22]. While repair mechanisms after a DSB normally maintain gene integrity, inappropriate repair can lead to disease. In the case of various lymphomas, it is known that the addition of a toxin or chemotherapeutic induced DSB to programmed DSBs for class switching leads to the cancer (80).

Why wouldn't the contaminating human fetal DNA injected with our vaccines be cleared by the presence of DNA digesting enzymes such as DNase? Clearance by plasma DNase is unlikely for several reasons : 1) first the concentration of plasma DNase is too low to clear the quantities of contaminating DNA that are injected, 2) the DNA is likely to be protected from DNase degradation because of the presence of chromatin and histones that are also likely to come through the manufacturing process into the final vaccine, and 3) the residual fetal DNA may become encapsulated in microsomes that protect the DNA from digestion.

The DNA contaminating our vaccines may be present in a form bound to histones and chromatin due to the manufacturing processes used for vaccine production, and our measurements, as well as Merck's reported measurements, of the residual DNA levels indicate it is present in a quantity too high for DNase digestion. While unbound or naked DNA can be digested by DNase, the suspected quantities and character of the residual DNA makes this an unlikely event. In vitro we know that digestion of DNA requires 1000 times the concentration of DNase compared to the DNA in order for complete digestion to occur within a several hour time period. Healthy adults have been measured to have only 26 +/- 9 ng/ml of DNase in their plasma. (126), 10 fold less than the amount of contaminating DNA per vaccine, and certainly not the required 1000 fold excess to guarantee complete DNA digestion. Also arguing against potential digestion of the residual contaminating DNA, plasma contains microvesicles (127) (table 2), at a concentration of 5-50 ug/ml in healthy individuals (128) (page 2), 1,000 times the levels of DNase. These microvesicles contain RNA and DNA and stabilize the RNA and DNA. (129). abstract and page 143 left column (and Gahan PB page 2, Ref 129 b and Kahlert discussion section first paragraph Ref 129c). One example of surprising nucleotide stability are miRNAs that have been demonstrated to be excellent cancer biomarkers. miRNAs are normally highly unstable, however, their stability in the bloodstream is due to their encasement inside exosomes, one kind of these microvesicle (128). Once inside a microvesicle, DNA is protected from digestion, no matter whether it is bound or unbound. Additionally, to our knowledge, the concentration of circulating DNase in young children has not been measured, and may well be significantly lower than adults. Injected contaminating DNA fragments which will escape DNase digestion could then be internalized by cells in the local environment, of particular concern if the internalizing cell were to be a stem cell, or be encapsulated and transported to the brain either by means of the bloodstream or by means of retrograde axonal transport.⁴²

How might the residual DNA reach the brain of a young child? Vaccination elicits an immune response with its concomitant physiological changes and cytokine expression, known to increase permeability across the blood-brain barrier. Furthermore, a pre-existing illness would predispose a child for diminished blood-brain barrier competence and therefore transport of contaminating DNA into the central nervous system. Alternatively, DNA-containing microvesicles can be transported retrograde to the brain by motor nerve proteins such as kinesin or its homologs, which are known to transport vesicles in axons (130) (page 1). Even non encapsulated DNA has been shown to be transported by kinesin retrograde through nerve axons to reach the central nervous system (131). page 5 and Thakor ref 131b page 2128 and ref 139 . Kinesin transports vesicles at about 1 um/sec (132), so contaminant DNA injected into an arm or leg muscle could be taken up by the nerve endings and reach a child's brain in a few days or as long as a few weeks. In the brain, DNA can then be incorporated into a brain cell's DNA by homologous or illegitimate recombination. The human neuron cell cycle length is 8-12 hours, and human cells incur 10^6 base lesions per day. Since the human brain contains 10^{10} - 10^{11} neurons, the spontaneous homologous recombination rate could be on the order of 1 per 10^6 every 8-12 hours (133), a potential rate significant enough to allow illegitimate or homologous recombination and subsequent associated de novo mutations (83) (3).

In concert with the presence of contaminating human DNA of appropriate size and sequence, factors which compromise the blood-brain barrier may be important determinants of the susceptibility of each individual child to the potential adverse effects of contaminating human DNA. The

⁴² See Oztas, E. Neuron Tracing published online, 1/31/03 Neuroanatomy.cog

measles and rubella viruses can cross the blood-brain barrier⁴³, so infection with certain viruses can compromise the barrier. Infection, whether viral or bacterial, can lead to compromised blood-brain barrier function. Infection status, in the absence of a blood test, could be indicated by surrogate measurements such as body temperature, presence of runny nose, stomach or intestinal upset, loose stools, or lethargy. Additionally, an immune response to the contaminating DNA can elicit an inflammatory response, also demonstrated to compromise the blood brain barrier. Studies have shown that DNA can elicit immune responses either as simple double stranded DNA (134), due to its methylation status (135), or as DNA bound to proteins (136). MRC-5 is known to be hypomethylated (Zelko 2010 ref 135b) and WI-38 has likewise been suggested to be hypomethylated (Huang 1999 ref 135c).

While there have been studies that have tried to address infection status and its relationship to autism diagnosis, these studies have, however, been too small to yield meaningful results and have not analyzed data relative to time of vaccination (137) (138). Unfortunately, the large, published population studies regarding autism have not discussed each child's infection status immediately prior to or after vaccination. These data may be critical to understanding why some children might be affected, but not others.

Although human gene therapy by intramuscular injection is still not in common use, studies in rats and mice have shown retrograde axon transport of intramuscularly injected and nerve ending injected DNA resulting in gene expression in the central nervous system or brain tissue (139) (57.4 trillion plasmids) (140) (10 million vectors) (141) (143) (144) (about 8-44 trillion plasmids). Additionally, multiple studies have demonstrated trans-synaptic transport⁴⁴ of nucleic acids retrograde to the central nervous system or the brain. In other words, nucleic acids taken up by nerve endings in the periphery of the body can be transported back up into the central nervous system and delivered across more than one nerve cell, ultimately being transported into the brain (145) (146) (147). As an example, in one of the cited references, transganglionic transport was demonstrated using nucleic acid expression of beta-galactosidase after injection into the tongue muscle of mice and expression was observed in the hypoglossal nucleus demonstrating retrograde transport, and also in the trigeminal tract and nucleus solitarius, indicating trans-synaptic transport of the nucleic acid once retrograde transport had occurred (148). Trans-synaptic transport has also been demonstrated by Hennig (2003 ref 148b) after injection of 15 million particles into the eye, gene expression was found 8-12 weeks later in the thalamus and tectum.

While infants were once thought to be born with the total number of nerve cells that they will have through life, we know now that cell death and replacement occur throughout postnatal life at a measurable level of approximately 0.002%, similar to the heart. Recent research has shown that potential ongoing neurogenesis occurs in the hippocampal region and the dentate gyrus (149) (review of adult neurogenesis documented in all mammals studied to date, including humans) (150). Indeed, abnormalities in hippocampal signaling induce autistic like phenotype in mice (Barnes ref 150b and Hammer ref 150c). Neurogenesis has also been demonstrated in adult brain, including humans, in the subventricular zones along the walls of the lateral ventricles (Fiorelli ref 150d). Extrapolating from evidence of thousands (10-17 thousand) of new neuroblasts in the young adult rat brain daily, with 100 billion nerve cells in the human brain, taking into account that turn-over takes less than 24 hours, we can estimate that 2 to 2.8 million replacement nerve cells are made each day. Furthermore, in addition to normal nerve cell death and replacement after birth, new glial cells are rapidly produced, new neuron synapse formation (cell-to-cell contact) continues to occur as we learn new things, pruning of unnecessary synapses continues for the first few years of life, and dendrite growth actively continues. Interestingly, imaging experiments of the brains of autistic children have shown abnormalities in the hippocampal region (151) (review page 5) and the dentate gyrus where ongoing nerve cell formation has been measured (Takarae ref 151b). Regressive autism could be triggered by retrograde transport and DNA insertion into a nerve cell in the thalamus, the dentate gyrus, the hippocampal region or into an astrocyte. Nervous system glial cells are critical for normal nerve cell function and communication and multiple sclerosis is one disease known to have disturbed glial cell function as one of the etiologies. In addition to the potential for DNA insertion to occur during new nerve cell generation, the ongoing base lesion repair process, mentioned previously, presents the opportunity for DNA insertions and mutations to disrupt synapse formation, as protein translation processes (152) may be disrupted by DNA insertion during base-lesion repair in a non-dividing cell.

We know that a few malfunctioning cells can result in the failure of an entire organ. For example, in animals and in humans, apoptosis at a level as low as 0.025% (control is 0.002%) leads to heart failure. (153) (page 567 of review) This is an accumulated heart cell death as low as 250,000 cells, from a normal heart with 2,000,000,000 (0.0125% of the total cell number) leading to heart failure and death. (154). Scientists interested in gene therapy have already demonstrated that in cell culture, spontaneous genomic incorporation of DNA fragments of 500 bp has an efficiency of 4% per hour. The smaller DNA fragments in vaccines should be taken up even more efficiently, and we can estimate that of the more than 1

⁴³ http://immunopaedia.org.za/fileadmin/pdf/Measles_virus_28Nov11.pdf, http://www.betterhealth.vic.gov.au/bhcv2/bhcarticles.nsf/pages/Viral_encephalitis

⁴⁴ retrograde transport within axons and dendrites of nerve cells, Schwab, M.E. et al., Retrograde axonal and transsynaptic transport of macromolecules: Physiological and pathophysiological importance, [Agents and Actions](#) September 1977, Volume 7, [Issue 3](#), pp 361-368.

trillion (1,000,000,000,000) human DNA fragments per vaccine, 40 million could be efficiently incorporated by cells within one hour of injection, and 320,000,000 fragments could be incorporated within 5 hours after injection (39).

MUTATIONS

Not only can small DNA fragments efficiently insert into the recipient's genome, they have also been shown to be involved in de novo mutations and genomic instability (53) (155) (156) (157). Indeed, the FDA in their "Guidance for Industry: Considerations for Plasmid DNA Vaccines for Infectious Disease Indications" states in section V.G. Biodistribution, Persistence, and Integration Analysis that "Theoretical concerns regarding DNA integration include the risk of tumorigenesis if insertion reduces the activity of a tumor suppressor or increases the activity of an oncogene. In addition, DNA integration may result in chromosomal instability through the induction of chromosomal breaks or rearrangements." (158). Complicating the field of trying to document small fragment DNA insertions in children who receive contaminated vaccines and suffer an encephalopathy as a result is the fact that DNA fragments can insert into a genome, induce subsequent mutations and then be excised from the genome, leaving the mutations behind. During the 1999 FDA workshop, this phenomenon was also discussed, particularly the observation that DNA can insert into a genome, cause mutations upstream or downstream of that insertion and then be excised from the genome, a phenomenon termed Hit-and-Run (7) (pages 61-75). The ability of exogenous DNA to 'Hit-and-Run' presents a complex problem of identifying DNA insertions associated with mutations, making access to databases such as the VSD for rigorous retrospective epidemiologic analysis more important. Despite the complexities of measuring genomic insertions because of this 'Hit-and-Run' phenomenon (which is mediated by DNA patrolling enzymes active constantly in every cell), insertions have indeed been documented in children with simplex autism.

Indeed, whole exome sequencing of DNA isolated from the peripheral blood of 20 children with autism spectrum disorder has identified non-inherited insertions and deletions in every sample examined (159). The exome is the portion of the DNA that codes for protein products and includes exons and their splice sites. Addition of a further 189 whole exome sequencing samples from children with ASD and their unaffected parents as well as 50 unaffected siblings demonstrated that 60% of the children had severe de novo genomic changes consisting of copy number variations, single nucleotide mutations and insertions/deletions (160). Particularly intriguing, in the O'Roak 2011 and 2012 publications, 806 rare insertions/deletions (indels) were documented in 189 ASD subjects (100%), and 18 of the total 209 (9%) children with an ASD had de novo indels in their exomes, while their unaffected siblings had 0 (0%) de novo indels (160) (supplementary Table 2). Of the 18 de novo indels, 16 led to truncated proteins, a severe genomic disruption event. Insertions/deletions are known to cause subsequent additional mutations (3) and therefore, this observation requires further studies to determine the DNA source for the de novo insertions and whether the DNA fragments contained in childhood vaccines may provide the DNA documented in de novo insertions.

Whole exome sequencing covers between 1% to 1.5% of the entire genome if 100% coverage of exomes is achieved. Typically coverage ranges from 35-93% using this sequencing technique. While exons are estimated to contain approximately 85% of inherited disease causing mutations (161) (162), 80% of the entire genome is biochemically active and between 8-20% of the genome codes for regulatory regions that modulate transcription of exon coding regions (163). Accordingly, a disruptive mutation in up to 20% of the genome could be expected to have a phenotypic functional effect. Indeed, in a meta-analysis of 7,000 viral insertions from various clinical trials, Deichman et. al. demonstrated that viral insertion led to a leukemic event not through direct mutagenesis primarily but through insertions in non-exomic sites that modulated gene transcription rate and activity (57) (page 7 of 9, second column, paragraph 1), and as reviewed by Williams and Baum, retroviruses are known to upregulate genes over distances as long as 10 kilobases or more (56).

We now know that contained within what has been called the 'non-protein' coding region of the human genome (the 'non-protein' coding region comprises 98% of the entire genetic code) are regions with potential functional roles. These regions include microRNAs (approximately 20 base pairs in length) and at least 5000 ultraconserved regions that are approximately 100 base pairs in length (164). There have been found to be as many as 2700 novel transcription starts in the 30Mb regions studied by the ENCODE project (165). MicroRNAs have been shown to be involved in gene regulation (166), (167), and some of the ultraconserved regions are located near regulatory sequences and are suspected of having regulatory functions. Coincidentally, a recent study has shown that some microRNAs that are dysregulated in the brains of autistic subjects are microRNAs that are involved in regulation of genes that have been implicated in autism (168). Therefore, exome sequencing most likely significantly underestimates the number of deleterious mutations present. While O'Roak et. al. detected de novo deleterious genomic changes in 60% of children with ASD who have no affected siblings (called simplex ASD), had they sequenced the entire genome rather than merely the exomes, most likely they could have detected multiple deleterious genomic changes in all of the children tested. Simplex ASD accounts for approximately 75% of the cases of ASD, while 25% have an affected sibling. Multiple additional publications have demonstrated the presence of hundreds of non-inherited, diverse mutations in the DNA of children with autism spectrum disorder (169) (170), (171), (172), (173), (160), (90).

CONCLUSION

The dangers of residual human diploid DNA and retroviral fragments are an unstudied risk to vaccine recipients, and yet, the overwhelming body of scientific literature demonstrates the high likelihood of auto-immune and/or insertional mutagenesis dangers from these contaminants. Without doubt, this is an issue that cries out for serious scientific and epidemiological investigation. However, for our purposes here, there can be no question that this is a plausible means by which the defect in these vaccines creates a well understood risk of doing significant damage to the human genome causing damage to the functionality of nerve cells and others. Thus, the vaccine can do the kind of damage seen in VJM and every other child whose parents have asserted this theory of causation. Combined with the results of our epidemiological study to date, it is more likely than not that the introduction of vaccines contaminated with human residuals, including DNA and retroviruses, is the environmental factor described by McDonald, et al, of the Environmental Protection Agency in “Timing of Increased Autistic Disorder Cumulative Incidence”, Ex. 27, as these vaccines have widespread exposure in developed countries. Their introduction or increase in dosage matches the timing of the epidemic and each increase in the rate of onset. There is a dose-response relationship. The above described toxic mechanisms that could affect early human development exist everywhere the vaccine is administered; providing a universal environmental trigger, absent prior to the autism epidemic, demonstrating a dose-response effect, and having established pathologic mechanism of action. Ex. 27, pp. 2115-2116. Thus, it is more likely than not that the MMR II vaccine did do this damage to VJM and other children.

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<https://www.soundchoice.org/open-letter-to-legislators/>

OPEN LETTER FROM DR. THERESA DEISHER TO LEGISLATORS REGARDING FETAL CELL DNA IN VACCINES

Posted on May 8, 2019

OPEN LETTER TO LEGISLATORS REGARDING FETAL CELL DNA IN VACCINES

April 8, 2019

My name is Dr. Theresa Deisher. I am Founder and Lead Scientist at Sound Choice Pharmaceutical Institute, whose mission is to educate the public about vaccine safety, as well as to pressure manufacturers to provide better and safer vaccines for the public. I obtained my doctorate from Stanford University in Molecular and Cellular Physiology in 1990 and completed my post-doctoral work at the University of Washington. My career has been spent in the commercial biotechnology industry, and I have done work from basic biological and drug discovery through clinical development.

I am writing regarding unrefuted scientific facts about fetal DNA contaminants in the Measles-Mumps-Rubella vaccine, which must be made known to lawmakers and the public.

Merck's MMR II vaccine (as well as the chickenpox, Pentacel, and all Hep-A containing vaccines) is manufactured using human fetal cell lines and are heavily contaminated with human fetal DNA from the production process. Levels in our children can reach up to 5 ng/ml after vaccination, depending on the age, weight and blood volume of the child. That level is known to activate Toll-like receptor 9 (TLR9), which can cause autoimmune attacks.

To illustrate the autoimmune capability of very small amounts of fetal DNA, consider this: labor is triggered by fetal DNA from the baby that builds up in the mother's bloodstream, triggering a massive immune rejection of the baby. This is labor.

It works like this: fetal DNA fragments[i] from a baby with about 300 base pairs in length are found in a pregnant mother's serum. When they reach between 0.46– 5.08 ng/mL, they trigger labor via the TLR9 mechanism[iii]. The corresponding blood levels are 0.22 ng/ml and 3.12 ng/ml. The fetal DNA levels in a child after being injected with fetal-manufactured vaccines reach the same level that triggers autoimmune rejection of baby by mother.

Anyone who says that the fetal DNA contaminating our vaccines is harmless either does not know anything about immunity and Toll- like receptors or they are not telling the truth.

If fetal DNA can trigger labor (a naturally desired autoimmune reaction), then those same levels in vaccines can trigger autoimmunity in a child. Fragmented fetal DNA contained in vaccines is of similar size, ~215 base pairs.^[iii]

This is direct biological evidence that fetal DNA contaminants in vaccines are not in low innocuous amounts. They are a very strong proinflammatory trigger.

Administration of fragments of human fetal (primitive) non-self DNA to a child could generate an immune response that would also cross-react with the child's own DNA, since the contaminating DNA could have sections of overlap very similar to the child's own DNA.

Children with autistic disorder have antibodies against human DNA in their circulation that non- autistic children do not have. These antibodies may be involved in autoimmune attacks in autistic children.^[iv]

Duke University demonstrated in a recently conducted study that significant improvements in behavior were observed when children with autism spectrum disorder were treated with their own banked autologous cord blood^[v]. This treatment clearly shows that most children with autism are not born with it since genetic diseases like Down syndrome or muscular fibrosis cannot be treated with autologous stem cells. Therefore, an environmental trigger, or triggers, introduced to the world around 1980 when autism first began to rise, must be identified and eliminated or reduced in the environment.

- Strong change-point correlation exists between rising autism rates and the vaccine manufacturing switch from animal-derived cell lines for rubella vaccine to human aborted cell lines in the late 70s^[vi].
- The earliest change point for Autistic Disorder (AD) birth year was identified for 1981 for California and U.S. data, preceded by a switch in the manufacturing process:
- In January 1979, the FDA approved the manufacturing switch for the rubella virus from animal based (high passage virus, HPV-77, grown e.g. in duck embryo cells) to the human fetal cell line WI-38 using the RA27/3 virus strain^[vii]. Both the newly approved monovalent rubella vaccine and a trivalent mumps, measles and rubella vaccine utilize the WI-38 fetal cell line for manufacturing of the rubella vaccine portion.
- Prior to 1980, autism spectrum disorder was a very rare, almost unknown disease. According to the figures of the CDC, the rate of autism in 2014 was 1 in 59 children, a very steep increase since just 2000, when it was 1 in 150. CDC: "The total costs per year for children with ASD in the United States were estimated to be between \$11.5 billion – \$60.9 billion (2011 US dollars)^[viii]."
- Recently, duplications and de novo deletions have been recognized in up to 10% of simplex autism spectrum disorders, corroborating environmental triggers on the genetics of autism spectrum disorders^[ix].
- The rubella portion of the MMR vaccine contains human derived fetal DNA contaminants of about 175 ngs, more than 10x over the recommended WHO threshold of 10 ng per vaccine dose^[x].
- No other drug on the market would receive FDA approval without thorough toxicity profiling (FDA follows international ICH guidelines) -> this was never conducted by the pharmaceutical industry for the DNA contamination in the MMR vaccine.
- Vaccines produced with human fetal cell lines contain cell debris and contaminating residual human DNA, which cannot be fully eliminated during the downstream purification process of the virus^[xi].
- Moreover, DNA is not only characterized by its sequence (ATCG), but also by its epigenetic modification (e.g. DNA methylation pattern etc.). This decoration is highly species specific, which is why non-human DNA will be eliminated through activation of TLR9 and consequent antibody production against the non-human DNA, while this is not necessarily the case with fetal human DNA.

Injecting our children with human fetal DNA contaminants bears the risk of causing two well-established pathologies:

1) Insertional mutagenesis: fetal human DNA incorporates into the child's DNA causing mutations. Gene therapy using small fragment homologous recombination has demonstrated that as low as 1.9 ng/ml of DNA fragments results in insertion into the genome of stem cells in 100% of mice injected^[xii]. The levels of human fetal DNA fragments in our children after vaccination with MMR, Varivax (chickenpox) or Hepatitis A containing vaccines reach levels beyond 1.9 ng/ml.

2) Autoimmune disease: fetal human DNA triggers a child's immune system to attack his/her own body.

An additional concern: retrovirus contamination.

Human endogenous retrovirus K (HERVK) is a contaminant in the measles/mumps/rubella vaccine^[xiii].

- HERVK can be reactivated in humans^[xiv]. It codes for a protein (integrase) specialized in integrating DNA into the human genome.
- Several autoimmune diseases have been associated with HERVK activity^[xv].

- It is also in the same family of retroviruses as the MMLV virus used in a gene therapy trial, in which inappropriate gene insertion (insertional mutagenesis) led to subsequent additional somatic mutations and cancer in 4 of 9 young boys[xvi].
- It is therefore possible that the HERVK gene fragment present in the MMR vaccine is active, codes for the integrase or the envelope protein, and thus has the potential to induce gene insertion, fostering insertional mutagenesis and autoimmunity.

The presence of both the high level contaminating fetal DNA as well as the HERVK contamination in the MMR vaccine is an unstudied risk with huge implications and dangers for individual and public health.

Solution: Pressure manufacturers to switch back to animal cell line derived rubella vaccines as was successfully done in Japan:

- Based on Takahashi strains of live attenuated rubella virus, produced on rabbit kidney cells. A single dose of this vaccine has been recently proven to retain immunity for at least 10 years when rubella was under regional control[xvii].
- Split MMR vaccine into three individually offered options as done in Japan.

The MMR vaccine manufacturing process needs to be changed to address and eliminate the above risks for the public.

Thank you for your consideration. I will be happy to address any questions you may have concerning the above.

Sincerely,

Theresa A. Deisher, Ph.D.

END NOTES

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The following links provide the video and PDF of the conference of 27.01.2019 (Rome) to which Dr. Deisher presented the recent results of her studies

https://drive.google.com/file/d/1drHvTXu56FjQ8ZJyuRi6keFosXI-r_pU/view

<https://www.youtube.com/watch?v=mvsyTYf4Dd4>

Data la rilevanza per la salute pubblica dei risultati scientifici supportati dalla ricerca della dr.ssa Deisher si richiede all'EMA quanto segue:

- di fornire la letteratura scientifica e gli studi effettuati dal produttore che dimostrano che le linee cellulari diploidi attualmente contaminanti i vaccini MPR ed MPRV, in particolare l'MRC-5, e le cellule embrionali di pollo, sono prive di attività mutagenica, oncogenica e tumorigenica sia in vitro che in vivo e non pongono alcun rischio per la salute dei vaccinati nel breve, medio e lungo termine.
- Di fornire la letteratura scientifica e gli studi effettuati dal produttore che dimostrano che i retrovirus endogeni ed esogeni riscontrabili nei vaccini contaminati da cellule diploidi non pongono alcun rischio per la salute dei vaccinati nel breve, medio e lungo termine.

Dossier EMA – NGS

Conclusioni finali sulla indagine sulla qualità

L'indagine condotta permette di affermare che i vaccini analizzati presentano vari tipi di criticità legate alla qualità, la sicurezza e l'efficacia dei vaccini e che ne emerge un quadro complessivo di uno scarso controllo della qualità delle materie prime e del prodotto finale che viene inoculato.

Ci rivolgiamo quindi all'EMA e a suoi esperti affinché esamini i nostri dati. Chiediamo inoltre di poter esaminare i file nativi delle repliche delle nostre analisi che ci auspichiamo vengano effettuate quanto prima sui vaccini in oggetto.

Poiché non mettiamo in dubbio la rigerosità del lavoro svolto dall'EMA nel garantire la qualità, sicurezza ed efficacia dei vaccini in commercio, confidiamo che i risultati da noi presentati vengano valutati in maniera approfondita per poter avere una risposta incontestabile ai quesiti che poniamo e per risolvere le criticità che abbiamo avuto modo di rilevare.