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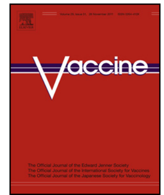

Transposon leads to contamination of clinical pDNA vaccine

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ABSTRACT

We report an unexpected contamination during clinical manufacture of a Human Papilomavirus (HPV) 16 E6 encoding plasmid DNA (pDNA) vaccine, with a transposon originating from the *Escherichia coli* DH5 host cell genome. During processing, presence of this transposable element, insertion sequence 2 (IS2) in the plasmid vector was not noticed until quality control of the bulk pDNA vaccine when results of restriction digestion, sequencing, and CGE analysis were clearly indicative for the presence of a contaminant. Due to the very low level of contamination, only an insert-specific PCR method was capable of tracing back the presence of the transposon in the source pDNA and master cell bank (MCB). Based on the presence of an uncontrolled contamination with unknown clinical relevance, the product was rejected for clinical use. In order to prevent costly rejection of clinical material, both in-process controls and quality control methods must be sensitive enough to detect such a contamination as early as possible, i.e. preferably during plasmid DNA source generation, MCB production and ultimately during upstream processing. However, as we have shown that contamination early in the process development pipeline (source pDNA, MCB) can be present below limits of detection of generally applied analytical methods, the introduction of "engineered" or transposon-free host cells seems the only 100% effective solution to avoid contamination with movable elements and should be considered when searching for a suitable host cell-vector combination.

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1. Introduction

Persistent infection with high-risk Human Papilomavirus (HPV) subtypes is strongly associated with the development of cancers of the cervix, vulva, vagina, penis, anus and oropharynx. One of the prerequisites for carcinogenesis is the continuous expression of the oncogenic early proteins E6 and E7 [1]. As E6 and E7 are strictly intracellular proteins, T-cell mediated immunity is needed to recognize the premalignant and malignant cells. Therefore, DNA vaccination with a plasmid encoding these oncogenes is an attractive immunotherapeutic approach for the induction of a HPV 16 specific cytotoxic T-cell response against HPV-induced lesions [2]. We developed an HPV16 E6 based DNA-vaccine composed of a pVAX backbone with an insert encoding a fusion protein composed of a signal peptide, a series of minimal CD4 helper epitopes, a gene-shuffled version of HPV16 and a C-terminal ER retention signal KDEL: pVAX sig-HELP-E6SH-KDEL further referred to as the HPV16 E6 vaccine [3]. In preclinical studies this HPV16 E6 vaccine proved to be highly effective and safe, and warranted clinical evaluation [4]. In an upcoming clinical study, HPV 16 E6 vaccine will be administered using the in-house developed tattoo strategy [5]. It was shown previously that this short-interval intradermal DNA vaccination leads to the rapid and sustained development of both T-cell and B-cell responses [6].

To generate the HPV16 E6 vaccine for clinical use, we set up a manufacturing process according to Good Manufacturing Practices (GMP) guidelines [7]. In summary, this process consists of the production of an E6 Master Cell Bank (MCB), production of E6 bulk drug from this MCB and subsequently production of E6 final product from E6 bulk drug [8]. The MCB, expanded from a single host cell transformed with the plasmid of interest, was established in order to enable the manufacture of sufficient pDNA bulk drug of consistent quality in time. The manufacture of bulk drug pDNA consists of the expansion by fermentation (upstream processing) of an MCB aliquot followed by purification of the pDNA product (downstream processing). In this process, contaminants like genomic DNA, RNA, proteins, and endotoxins are reduced to predefined, acceptable levels. Production of pDNA final product involves an aseptic fill and finish process resulting in the desired pharmaceutical dosage form containing the pDNA of interest in

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the required dose unit. All abovementioned production steps are monitored by various in-process controls, focused on pDNA concentration and purity during processing [8]. MCB, pDNA bulk drug and final product are subject to extensive quality control testing (also in-process) before release for (clinical) use. Several papers have been published on the (small-scale, ≤10 L) manufacture of both non-clinical and clinical grade plasmid DNA [8–13].

Saedler et al. already reported in 1973 multiple copies of the IS2 element in *Escherichia coli* [14]. Fifteen years later, the spontaneous insertion of an IS2 element was described by de Togni et al., when spontaneous insertion of an IS2 element into the plasmid pUC 18 had occurred. IS2 insertions also resulted in incorrect annotation of genes and proteins from many different species. A detailed search of GenBank showed that the IS2 is present within many eukaryotic nucleotide sequences and it is likely to be incorporated into the insert during the cloning process [15]. IS elements can act as genomic parasites, and play a role in promoting the evolutionary adaptation of their hosts and they are able to generate some of the beneficial mutations that increase organism fitness [16].

Multiple sites of IS2 integration have been identified, in example Oliveira et al. have found IS2 transposition upstream of the kanamycin resistance gene in a pDNA vaccine vector (pCIneo::IS2). pCIneo spontaneously recombines due to the presence of two 28 bp direct repeats[17]. Certain regions of the target genome serve as so-called "hot spots" (e.g., repeated sequences) for integration [18]. For example, regions of homology with an internal sequence of IS2 and a pentanucleotide GGTAT sequence might be involved. Also the site of insertion is identified as pentanucleotide ATACC. Insertion likely occurs during growth of a single colony and segregation of the progeny plasmids [18].

We report an unexpected contamination of the HPV16 E6 bulk drug, which appears to originate from the introduction of a host cell transposon in the pDNA vector.

2. Materials and methods

2.1. E6 source pDNA

The sig-HELP-E6SH-KDEL insert was constructed by standard molecular cloning techniques and cloned into the pVAX1 backbone (No. V260-20, Invitrogen, Grand Island, NY, USA) using HindIII and XbaI at The Netherlands Cancer Institute (NKI-AVL, Amsterdam, The Netherlands). Subsequently, the pVAX sigHELP-E6SHKDEL plasmid of 4.1 kb with a kanamycin resistant marker (E6 vaccine) was produced using *E. coli* DH5α as host cell and purified with the Qiagen 'mega' endotoxin-free plasmid purification kit (Qiagen, Venlo, the Netherlands) to obtain HPV16 E6 source pDNA. Before further use, the obtained HPV16 E6 source pDNA was analyzed for purity and identity by agarose gel electrophoresis (AGE), restriction analysis and sequencing.

2.2. E6 MCB and bulk drug production

All production steps were performed under cleanroom conditions (GMP EU class A with class B background) [19] *E. coli* DH5 cells were used for transformation (ATCC #53868, Teddington, Middlesex, UK). Cells were made competent with $CaCl_2$ and subsequently transformed with E6 using a standard heat shock method [20]. These cells were plated on Luria Bertani (LB) plates containing 100 μg/mL kanamycin (Biotrading Benelux, Mijdrecht, The Netherlands) and grown. One single colony was isolated and grown in 175 mL LB-Miller broth (Sigma–Aldrich Chemicals BV. Zwijndrecht, The Netherlands) containing 100 μg/mL kanamycin (Roche diagnostics Nederland BV. Almere, The Netherlands) at 37 °C in a 1 L sterile baffled shake flask (Nalgene, Rochester, NY, USA)

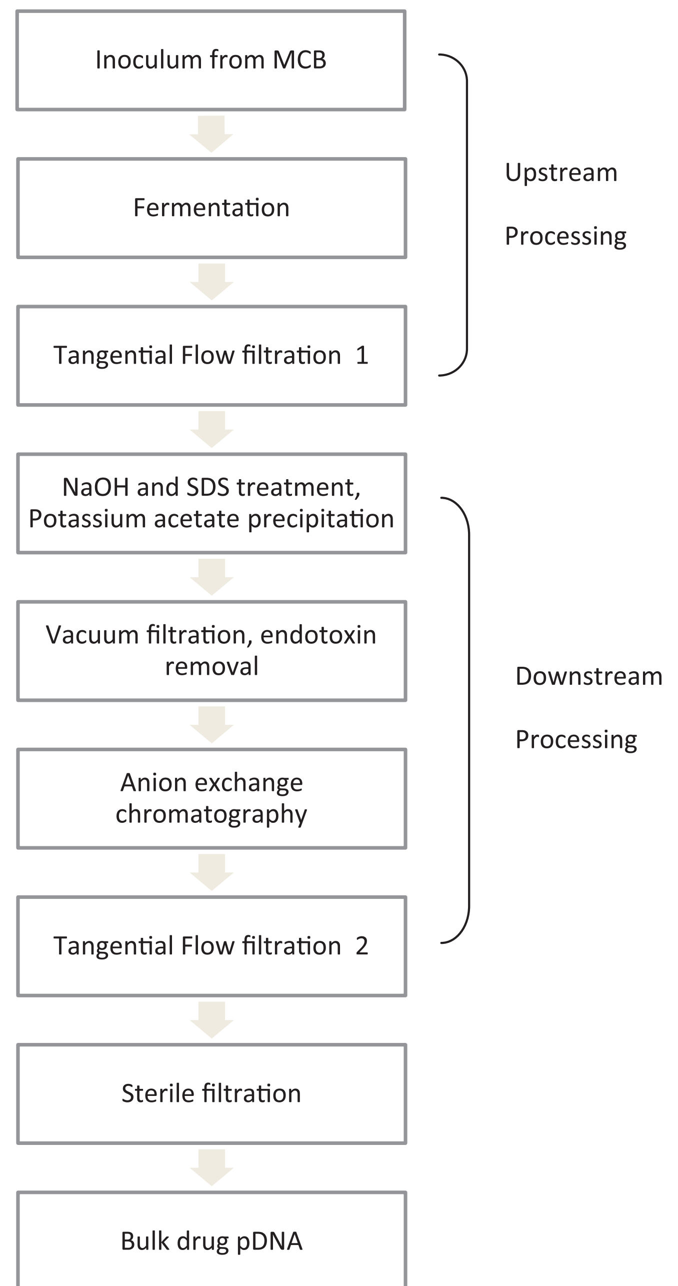

Fig. 1. Process flow sheet for the production of E6 bulk drug.

at 200 rpm. When an $OD_{600} \geq 0.7$ was reached, glycerol (BUFA, Uitgeest, The Netherlands) was added to the culture (30% v/v). Aliquots of 1 mL were frozen at −80 °C. One MCB lot was produced. Quality control of the MCB was performed according to European Pharmacopoeia (Ph. Eur.) [21] and FDA guidelines [22,23]. Plasmid identity was confirmed by sequencing and restriction analysis.

The production process of E6 bulk drug is summarized in Fig. 1. The culture grown from one MCB vial was inoculated in a BioFlo 3000 benchtop fermentor with a working volume of 10 L (New Brunswick Scientific BV, Nijmegen, The Netherlands) containing 7 L growth medium (KH_2PO_4, K_2HPO_4 $(NH_4)_2SO_4$ and glycerol (14 g/L)) feed supplement (4 g/L/h), 58.1 mL medium supplement solution (thiamine·HCl and $MgSO_4{\cdot}7H_2O$ in WFI), 7 mL trace element solution ($FeCl_3{\cdot}6H_2O$, $ZnCl_2$, $CoCl_2{\cdot}6H_2O$, $Na_2MoO_4{\cdot}2H_2O$ $CaCl_2$, $CuCl_2{\cdot}2H_2O$ and H_3BO_3 in 1.2 N HCl) and 50 mg/mL kanamycin at 37 °C under fed-batch conditions [24]. pH, dissolved oxygen (DO)

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