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Short communication

Isolation of influenza viruses in MDCK 33016PF cells and clearance of contaminating respiratory viruses

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ABSTRACT

This paper summarizes results obtained by multiplex PCR screening of human clinical samples for respiratory viruses and corresponding data obtained after passaging of virus-positive samples in MDCK 33016PF cells. Using the ResPlexII v2.0 (Qiagen) multiplex PCR, 393 positive results were obtained in 468 clinical samples collected during an influenza season in Germany. The overall distribution of positive results was influenza A 42.0%, influenza B 38.7%, adenovirus 1.5%, bocavirus 0.5%, coronavirus 3.3%, enterovirus 5.6%, metapneumovirus 1.0%, parainfluenza virus 0.8%, rhinovirus 4.1%, and respiratory syncytial virus (RSV) 2.5%. Double infections of influenza virus together with another virus were found for adenovirus B and E, bocavirus, coronavirus, enterovirus and for rhinovirus. These other viruses were rapidly lost upon passages in MDCK 33016PF cells and under conditions as applied to influenza virus passaging. Clinical samples, in which no influenza virus but other viruses were found, were also subject to passages in MDCK 33016PF cells. Using lower inoculum dilutions than those normally applied for preparations containing influenza virus (total dilution of the original sample of $\sim 10^4$), the positive results for the different viruses turned negative already after 2 or 3 passages in MDCK 33016PF cells. These results demonstrate that, under practical conditions as applied to grow influenza viruses, contaminating viruses can be effectively removed by passages in MDCK cells. In combination with their superior isolation efficiency, MDCK cells appear highly suitable to be used as an alternative to embryonated eggs to isolate and propagate influenza vaccine candidate viruses.

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1. Introduction

Influenza virus isolation for monitoring epidemic influenza activity and for the selection of candidate vaccine strains has traditionally been conducted by cultivation in embryonated hen's eggs. Due to receptor limitations, such egg passaging can cause adaptive mutations of the haemagglutinin [1,2]. These egg-adaptive mutations do not revert on subsequent passage in mammalian cells, and they may alter the antigenic properties of the receptor binding site, which is also a critical binding site for virus inhibiting and protective antibodies [3,4]. In contrast to egg-passaged virus, mammalian cell-grown influenza virus preserves the sequence of the original human clinical sample.

During the last decade the worldwide National Influenza Centres have almost completely changed influenza virus isolation from egg culture to cell culture, mainly using MDCK cells. This change to cell culture was stimulated not only by the relative ease of conducting multiple isolations in cell cultures but also by the better antigenic match of MDCK-isolated viruses with field strains. Increasing difficulties in recovering isolates from embryonated eggs, particularly of H_3N_2 subtypes, has also contributed to the change to cell culture [5].

Several companies are currently developing cell culture-based influenza vaccines [6] and the first of those vaccines, produced in MDCK and Vero cells, have been licensed and distributed as interpandemic trivalent and pandemic H_1N_1 vaccines. Using the conventional, recommended reference viruses, these vaccines still originate from egg-derived virus isolates or the corresponding high-growth reassortants. Regulatory concerns, mainly with regard to the introduction of adventitious agents, are raised if candidate vaccine strains are derived directly from uncharacterised and uncontrolled cell lines. Collaborative studies have been initiated to investigate the growth and yield of influenza viruses in different cell lines, the efficiency and fidelity of influenza virus isolation, and the suitability for vaccine manufacture of different cell substrates [7]. Growth studies with a wide range of potentially contaminating viruses have been conducted and risk assessments have been made, comparing egg-derived and cell-passaged influenza viruses

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with regard to the risk of carrying adventitious viruses into vaccine manufacturing processes [8,9]. These assessments indicated that, in comparison to manufacturing in embryonated eggs, the introduction of Vero cells increases the risk of transmitting various viruses into the vaccine process, whereas the use of MDCK cells reduces the overall risk. Due to their limited permissiveness to viral growth, MDCK cell exert the same filter effect for human adventitious viruses as do embryonated eggs.

Here, we assess on the presence of co-isolated viruses in influenza virus isolates recovered from MDCK cells. This article provides more specific data about the kind and frequency of co-infecting respiratory viruses in human influenza virus-containing samples and about the fate of such co-infecting viruses during passage in MDCK cells.

2. Materials and methods

2.1. Clinical material

Nasal or pharyngeal samples from the 2007/2008 influenza season were provided by a clinical diagnostic laboratory located in Stuttgart, Germany. These samples from patients with acute respiratory tract infections were obtained by physicians mainly from Southern Germany and were sent to the diagnostic laboratory in liquid virus transport medium. Aliquots of the clinical specimens (with a laboratory number as an anonymous identifier) were sent to Novartis Vaccines in Marburg, Germany, by a weekly courier service. During transportation the samples were stored at 2–8 °C. Directly after receipt of the samples, MDCK 33016PF cells were inoculated (details see further below) with sample material. The cultures were harvested after 3 days of incubation, and the cell-free supernatants were aliquoted and stored at ≤−60 °C until further use.

2.2. Cell cultures and virus passaging

MDCK 33016PF suspension cells from Novartis working cell bank were cultivated in 500 ml disposable spinner flasks (Corning) in CDM medium, a chemically defined growth medium used for cell propagation (MDCK 33016 CDM, Lonza) and passaged at 3–4-day intervals. During those 3–4 days the cells grew from an initial seeding density of 1×10^5 cells/ml to densities between 1.0 and 1.5×10^6 cells/ml. For infections 4.5 ml cells were seeded in 50 ml filter tubes (TPP, Transadingen, Switzerland) at a cell density of $0.8–1.2 \times 10^6$ cells/ml. Cells in CDM medium were diluted at a 30/70% ratio into MDCK 33016 PFM medium ("protein-free medium", Gibco Invitrogen) supplemented with 0.5% of a penicillin/streptomycin solution (Sigma) and 900 IU/ml trypsin. To obtain a total culture volume of 5 ml, the added viral inoculum was diluted in 0.5 ml infection medium and was pre-diluted by several $\log_{10}$ steps, starting with a total dilution of at least 1:100. Inoculated cultures were then incubated at 33 °C for 3 days in a 5% CO_2 atmosphere in a ISF-1-W shaker incubator (Kuhner, Birsfelden, Switzerland). For virus harvests the cells were separated by centrifugation (800–1000 × *g* for 10 min) and the supernatant was recovered. Unless used freshly, e.g. for haemagglutination tests and subsequent passaging, aliquots of the supernatant were frozen at ≤−60 °C.

Haemagglutination (HA) testing was done with harvested material to define the starting material for the next passage. HA testing was performed in U-bottom microwell plates (Greiner Bio-One) using 100 μl of a serial $\log_2$ dilution in PBS (pH 7.0) of the test samples and 100 μl chicken or guinea pig red blood cells (0.5% in PBS pH 7.0). Results were read after 60 min (chicken erythrocytes) or 120 min (guinea pig erythrocytes) incubation at ambient temperature within a temperature range of 19–25 °C. Two different kinds of red blood cells were used since the actual H_3N_2 influenza strains did not react with chicken red blood cells. Material from the highest $\log_{10}$ inoculum dilution, which showed a clearly positive HA reaction after the previous passage, was used for the following passage.

Table 1
ResPlex II v2.0 multiplex PCR target pathogens and target genes.

Pathogen	Targeted gene
Respiratory syncytial virus A	Nonstructural protein (NS)
Respiratory syncytial virus B	Nonstructural protein (NS)
Influenza virus A	Nonstructural protein (NS)
Influenza virus B	Nonstructural protein (NS)
Parainfluenza virus 1	Nucleocapsid protein (N)
Parainfluenza virus 2	Nucleocapsid protein (N)
Parainfluenza virus 3	Nucleocapsid protein (N)
Parainfluenza virus 4	Nucleocapsid protein (N)
Human metapneumovirus	Fusion protein (F)
Rhinovirus	5′ UTR
Enterovirus (Coxsackie virus, Echovirus)	5′ UTR
Coronavirus OC43	Nucleocapsid protein (N)
Coronavirus 229E	Nucleocapsid protein (N)
Coronavirus NL63	Nucleocapsid protein (N)
Coronavirus HKU1	Nucleocapsid phosphoprotein (N)
Bocavirus	Nonstructural protein (NS)
Adenovirus types 3, 7, and 21	Hexon
Adenovirus type 4	Hexon

2.3. Multiplex PCR

Extraction of viral DNA or RNA from clinical specimens and culture supernatants was performed with the Nucleic Acid Isolation Kit I in the MagNA Pure compact extraction system (Roche) or with the QIAsymphony® Virus/Bacteria Midi Kit (Qiagen) in the QIAsymphony robotic system.

The ResPlex II v2.0 multiplex PCR panel (Qiagen) was used according to the manufacturer's instructions. The test applies a RT-PCR (reverse transcription and PCR reaction) by the OneStep RT PCR Kit (Qiagen) in combination with two pairs of specific primers for each target. The enzyme mix contains the Omniscript™ and Sensiscript™ reverse transcriptase and the HotStarTaq™ DNA polymerase. The dNTP mix contained 10 mM of each dNTP. The primer mix consisted of a mixture of individual primers for each viral target, carrying a tail with the target sequence for the superprimers, and the forward and backwards superprimers.

Results of the multiplex PCRs were read with the LiquiChip detection system, which consists of microspheres coated with target-specific hybridization molecules and a steptavidin–biotin based fluorescence detection reaction giving an individual fluorescence color pattern for each viral target. Result readings were evaluated with the QIAplex MDD-RVO Beta software. According to the manufacturer's instructions signals above values of 150 are positive, values below 100 are negative and values between 100 and 150 are considered as questionable results. The method's results are given as counts (median fluorescence intensity, MFI) but the method is not intended or designed to be used quantitatively.

The ResPlex II v2.0 method is designed to detect 18 different virus species or virus subgroups simultaneously. These pathogens and the target genes used are summarized in Table 1.

Independent, conventional in-house qRT-PCRs or commercially available PCR methods were used to confirm ResPlex results with clinical specimens. These methods and according references are summarized in Table 5.

Table 2
Number and distribution of virus positive results in human samples collected during the influenza season in spring 2008.

Virus	Positive results	Percentage
Influenza A	165	42.0%
Influenza B	152	38.7%
Adenovirus	6	1.5%
Bocavirus	2	0.5%
Coronavirus	13	3.3%
Enterovirus	22	5.6%
HMPV	4	1.0%
Parainfluenza virus	3	0.8%
Rhinovirus	16	4.1%
RSV	10	2.5%
Total	393	100.0%

3. Results

The total number of samples investigated was 468. Positive results with the ResPlex II v2.0 PCR were obtained with 370 (79%) samples. Due to 21 double and one triple infection in the same sample the total number of virus-positive results was 393 in the 370 samples. Of the positive results 317 (85.7%) were positive for influenza virus with an almost equal distribution between A and B subtypes. 76 positive results with 66 samples indicated the presence of other respiratory viruses. The proportion of the different viruses found by the multiplex PCR is shown in Table 2.

Of particular interest were those specimens in which a double infection of an influenza virus together with other viruses was detected. In 13 samples 14 positive (and 2 questionable) results for other viruses were found associated with influenza virus. These associated viruses are listed below along with extra remarks about 2 samples that gave questionable results (100–150 MFI).

- Adenovirus B and E – 2 samples.
- Bocavirus – 1 sample (plus 1 questionable bocavirus result in another sample).
- Coronavirus NL 63, OC43, HKU1 – 5 samples (plus 1 questionable coronavirus 229E in a sample found positive for influenza and enterovirus).
- Enterovirus – 3 samples (1 of which was positive for influenza-, entero-, and rhinovirus).
- Rhinovirus – 3 samples (1 of which was positive for influenza-, entero-, and rhinovirus).

These samples were passaged up to five times in MDCK 33016 PF cell as described in Section 2. In addition, sample 750 (compare Table 3) was also used for these passages, as it was questionably positive for bocavirus and contained influenza B. One other sample (sample 670, positive for coronavirus HKU1 in association with influenza virus B in the clinical specimen) could not be cultivated because there was not sufficient material.

As shown in Table 3, the only virus that was detectable after 2 (or 5) passages was influenza virus; the other contaminating viruses were lost during passage. The table also lists the total dilution of the original sample until passage 2 (10^{-7} to 10^{-9}) and passage 5 (10^{-22} to 10^{-28}). Only one sample (see sample 608 in Table 3), in which no virus could be recovered was passaged at lower dilutions. The order in which the detected viruses are listed in Table 3 reflects the counts found in the ResPlex method. Most co-infecting viruses had lower counts than the influenza virus. Sample 635 had highest counts for an enterovirus and similar counts for rhinovirus and influenza virus, sample 608 had higher counts for adenovirus than for influenza virus. However, it should be noted that the ResPlex method is not a quantitative method.

In a similar way, samples with positive and questionable multiplex PCR results only for viruses other than influenza virus were also cultivated for 2 or 3 passages in MDCK 33016PF cells. As shown in Table 4, only two passages usually were sufficient to eliminate the virus, so that almost all samples tested negative. Only three of the 54 viruses detected in the original sample still gave a very weak Resplex signal after the second cell culture passage: one coronavirus with a signal just above the questionable level and an enterovirus and one RSV at the questionable level. Considering the total dilution from the original sample to the second passage of only 2×10^4, it is possible that the original sample contained more than 10^4 viruses and remained (weakly) positive during 2 passages without any virus growth. When tested after the third culture passage (representing a 1:10 dilution of the clinical sample, these three samples tested negative by Resplex II, indicating no virus growth and that the weakly positive test results from the 2nd passage were obviously due to residual virus from the original clinical sample.

Table 5 shows the results of confirmatory test of clinical specimens using independent, conventional PCR methods.

4. Discussion

Influenza virus reference seeds are produced by WHO on an annual basis to match drifting influenza strains [19]. Reference viruses are released to vaccine manufacturers after the WHO recommendations have been published. These viruses are not subject to any specific testing for adventitious viruses. The corresponding vaccine must be manufactured, tested and distributed within only a few months in order to meet vaccination schedules [20–22]. Because of this short timeline, conventional broad spectrum testing of the influenza virus seed for adventitious agents cannot be performed in time, particularly if one considers that months may be needed to prepare virus from an independent source and specific antibodies against the same to neutralise the influenza virus. For conventional egg-derived viral seeds it is commonly assumed and supported by historical safety records, that many adventitious viruses are removed by egg passages. Because cell-derived influenza virus isolates are now being considered for use as starting material for vaccine manufacture, information is needed about the behaviour of adventitious viruses during cultivation of influenza viruses in suitable cell substrates. Our studies contribute such information for a cell line that is qualified for influenza vaccine manufacture.

The result presented here should be seen in context with specifically designed growth studies with a wide range of potentially contaminating viruses, which, along with the results of a systematic literature search on growth of viruses in MDCK cells, have been published previously, [8,9]. In those studies a standard amount of 10^6 infectious units (TCID50) per 100 ml culture was inoculated into MDCK 33016 cells and the cells were grown for at least 14 days (21 days for slow-growing viruses) in CDM growth medium. High dilution passaging was avoided but samples of suspended cells and medium were taken at regular intervals to be tested for the virus, and an adequate amount of fresh medium was added after sampling to maintain cell growth. The agents studied included: three human adenovirus (types 1, 5, 6), herpes simplex virus (HSV), Epstein–Barr virus, cytomegalovirus, parainfluenzavirus 3 and SV-5, respiratory syncytial virus (RSV) type A and B, human coronavirus 229E, human enterovirus species (Coxsackie A16, Coxsackie B30, Echovirus 6, poliovirus type 1), two human metapneumo virus strains, three different rhinoviruses, mammalian reovirus-3, BK polyomavirus, simian virus 40 (SV-40), budgerigar fledgling disease polyomavirus, avian C-type retrovirus (Rous sarcoma virus), avian infectious bursal disease birnavirus, two avian reovirus strains, minute virus of mice (MVM) parvovirus and porcine circovirus. Furthermore, the

Table 3
MDCK passages of field samples positive for influenza virus plus other viruses.

Sample no.	Original clinical sample	2nd passage MDCK 33016PF		5th passage MDCK 33016PF	
	Viruses detected	Virus detected	Total sample dilution	Virus detected	Total sample dilution
500	Influenza A, Enterovirus	Influenza A	10^{-8}	Influenza A	10^{-27}
521	Influenza A Adenovirus B	Influenza A	10^{-9}	nd	–
533	Influenza A, Bocavirus	Influenza A	10^{-9}	Influenza A	10^{-24}
559	Influenza A, Enterovirus (Corona 229E)[a]	Influenza A	10^{-9}	Influenza A	10^{-25}
565	Influenza B (Corona NL 63)[a]	Influenza B	10^{-7}	Influenza B	10^{-22}
608	Adenovirus E, Influenza A	none	10^{-4}	nd	–
621	Influenza B, Corona NL 63	Influenza B	10^{-9}	Influenza B	10^{-26}
635	Enterovirus, Influenza A, Rhinovirus	Influenza A	10^{-9}	Influenza A	10^{-23}
639	Influenza A, Corona OC43	Influenza A	10^{-9}	Influenza A	10^{-26}
664	Influenza A, Rhinovirus	Influenza A	10^{-9}	Influenza A	10^{-25}
721	Influenza A, Rhinovirus	Influenza A	10^{-9}	Influenza A	10^{-24}
750	Influenza B (Bocavirus)[a]	Influenza B	10^{-4}	Influenza B	10^{-13}
818	Influenza B, Corona HKU1	Influenza B	10^{-9}	Influenza B	10^{-28}

nd: not done.
[a] Questionable result placed in brackets.

Table 4
MDCK 33016PF passages of field samples tested positive or questionable for respiratory tract viruses.

Viruses detected (no. of samples)		
Original clinical sample	2nd passage MDCK 33016PF	3rd passage MDCK 33016PF
Enterovirus (16)	None (15), Enterovirus (1, questionable)	None (16)
Rhinovirus (12)	None (12)	nt
Coronavirus (9)	None (8), Coronavirus (1, borderline)	None (9)
Resp. Syncytial Virus (8)	None (7), RSV (1, questionable)	None (8)
Adenovirus (3)	None (3)	nt
Human metapneumovirus (3)	None (3)	nt
Parainfluenza virus (2)	None (2)	nt
Bocavirus (1)	None (1)	nt

Three double infections were counted individually. Maximum dilution of the original specimen for the 2nd passages was 2×10^4 3rd passage was done 1:10 from 2nd passage supernatant. nt: not tested.

Table 5
Confirmatory tests using independent, conventional PCR methods.

Target virus	PCR method [Reference]	ResPlex results confirmed	Remark
Influenzavirus	[10]	317/317	
Adenovirus	Euroclone Duplicα Real-time Adenovirus Kit	6/6	
RSV A, B	[11,12]	10/10	
Enterovirus	Artus® Enterovirus LC RT-PCR Kit version 2 [13]	9/22	Conserved 5′ UTR regions used for the ResPlex method are shared by both virus groups
Rhinovirus	[14,15]	11/16	
Parainfluenza virus 3	Novartis Vaccines & Diagnostics in-house realtime RT-PCR	0/3	From one sample no material was left for confirmatory test
Coronavirus NL 63	[16]	5/5	Includes one questionable ResPlex result (sample 656 in Table 3)
Coronavirus OC 43	[16]	1/1	
Cornavirus 229E	[16]	4/5	Includes one questionable ResPlex result (sample 559 in Table 3)
Coronavirus HKU1	[16]	3/3	
Bocavirus	[17]	3/3	Includes one questionable ResPlex result (sample 750 in Table 3)
hMPV	[18]	3/3	

growth of *Mycoplasma hyorhinis* and *Chlamydia trachomatis* were assessed. In those studies high virus growth was observed for parainfluenzavirus 3, SV5 and herpes simplex virus, slow growth was seen with mammalian reovirus 3, and questionable results (very low or no growth) were noted for the two avian reovirus. No growth was observed for the other viruses and agents tested. Infectious titers declined and were in most cases no longer detectable after 3–5 days of cultivation. Only for few very stable viruses, such as SV-40, virus titers persited longer.

Based upon those studies, and supported by the results of a systematic literature search (applicable to standard adherently growing MDCK cells), MDCK 33016 suspension cells support the growth of only a limited range of viruses. In this context the relevant viruses are influenza virus, parainfluenza virus, reovirus, and herpes simplex virus. This permissiveness spectrum is very similar to that seen in embryonated eggs [7]. Therefore, like embryonated eggs used in current influenza vaccine manufacture, MDCK 33016 cells should act as an effective barrier for a wide range of adventitious agents. Moreover, MDCK 33016 cells do not support the replication of many avian viruses. This is of particular relevance if an avian virus contaminant is introduced into the process by prior passaging of the vaccine virus strain in embryonated eggs.

The clinical specimens used for our studies were collected during the peak of an influenza season in February and March 2008 in order to gain more information about isolation rates in MDCK 33016PF cells in suspension culture. The results of those studies will be published elsewhere. Considering the selection of specimens, the high percentage of influenza-positive results is not surprising, but a significant number of samples (66/370 or 17.8%) also tested positive for other viruses, such as adenovirus, bocavirus, coronavirus, enterovirus, metapneumovirus (HMPV), parainfluenza virus (PIV), rhinovirus, and respiratory syncytial virus (RSV). Except for RSV and PIV, the same viruses were also detected as co-infections together with influenza virus. Such co-infections together with influenza viruses have also been published previously for RSV [23–25], PIV [23], HMPV [25,26], and for adenovirus and bocavirus [24], although the overall frequency was comparatively low. Those previous reports were all based on PCR detection methods, applying a more restricted virus spectrum than the ResPlex II method. We were unable to find reports about co-infections of influenza virus with other viruses identified via cell culture isolation methods, although such double-infected study materials have certainly been used in high numbers. This indicates that cell cultures selectively support replication of specific viruses and that, in addition, the virus identification methods used were less specific or less sensitive than PCR-based methods.

For the purpose of our studies the ResPlex II® multiplex PCR method was chosen because it combined detection of a wide range of relevant respiratory viruses with simple application (one-tube assay, rapid results from only one test run), and particularly because it could be applied to the available small volumes. Currently, limited information is available about the sensitivity and specificity of the ResPlex II v 2.0 test kit. We have compared the novel ResPlex III assay and existing techniques for the detection and subtyping of influenza virus during the influenza season 2006–2007 [27]. The methodology must necessarily make some compromises, for example, with regard to amplification conditions during the first cycles with specific primers. Thus it is not expected that sensitivity will be the same as that of monoplex PCRs. When compared to an in-house quantitative real-time PCR for influenza virus (detection limit 1–10 $TCID_{50}$/ml of a fresh influenza virus harvest), the ResPlexII v2.0 test appeared to be about 1 $\log_{10}$ step less sensitive.

The majority of positive results obtained with the ResPlexII v2.0 test could be confirmed by other, independent conventional published, in-house qRT-PCRs or commercially available PCR methods which used other target regions of the viral genomes. This applies to all 317 influenza positive samples, 10 of 10 RSV A and B positive samples tested, 6 of 6 adenovirus positive samples, 3 of 3 bocavirus positive samples (including one questionable ResPlex result), and 13 of 14 positive coronavirus samples (including 2 questionable ResPlex results). Differences were found for 2 parainfluenza virus 3 samples, for which ResPlex results could not be confirmed; likewise only 11 of 16 rhinovirus samples and 9 of 22 enterovirus samples tested negative in independent PCRs, but were positive with the ResPlex method. It remains to be determined whether the observed discrepancies are weaknesses of the ResPlex system or of the other, independent PCRs. However, the manufacturer of the ResPlex method confirmed certain cross-reactivities between enteroviruses and rhinoviruses, which have conserved 5′ UTR regions that were used as targets for the PCR primers. Since it is known that reovirus may grow in MDCK cells [9], we also screened many samples with an in-house reovirus qRT-PCR specific for mammalian orthoreovirus 1-3 (conserved region of the L3 inner capsid gene). Samples in which no other virus was detected by the ResPlex method were preferably used for the reovirus PCR. No reovirus was found in 271 of the specimens for which sufficient material was still available.

Whereas the specific virus growth studies summarized and discussed further above applied cell-culture adapted virus strains, the studies reported here used unadapted field virus strains and technical conditions as applied for influenza virus isolation and passaging. These studies confirmed that isolating influenza viruses in MDCK 33016PF cells effectively reduced co-infecting viruses. After only two passages and a 10^{-7} to 10^{-9} total dilution of the original specimen, adeno-, boca-, corona-, entero-, and rhinoviruses were no longer detectable. Only influenza viruses were recovered and remained the only detectable virus upon further passage. High dilutions of the inocula and short incubation periods of only 3 days until harvest or the next passage are conditions that are normally applied to effectively grow influenza viruses. These conditions certainly contributed to the rapid loss of the contaminating viruses. Only viruses that are present at very high titers and which grow very rapidly without adaptation would be able to survive such passaging.

In a second series of passages we also monitored more than 50 specimens that did not contain an influenza virus but were positive for other respiratory viruses. In these specimens interference by competing influenza virus growth was excluded. The culture conditions differed, as lower inoculum dilutions were used. Each sample/harvest was diluted 1:100 into the culture, which is the lowest standard dilution applied to recover very low-titred influenza virus. Also under these conditions 54 positive results for 8 different viruses became negative after only 2 or 3 passages and after a total dilution of the original specimen by a factor of 2×10^{-4} to 2×10^{-5}. When similar passages were conducted with adherent Vero cells ("Vero WHO seed"), several positive samples (adenovirus, rhinovirus, enterovirus, metapneumovirus, and bocavirus) remained positive after 2 passages. However, except for adenovirus, the counts did not increase but dropped (data not shown).

These results demonstrate that, under practical conditions as applied to grow influenza viruses, contaminating viruses can be effectively removed by passaging in MDCK 33016PF cells. In combination with their superior isolation efficiency [7,28], MDCK cells appear highly suitable to be used as an alternative to embryonated eggs to isolate and propagate candidate vaccine viruses.

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