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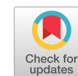
Environmental contaminant mixtures modulate *in vitro* influenza infection



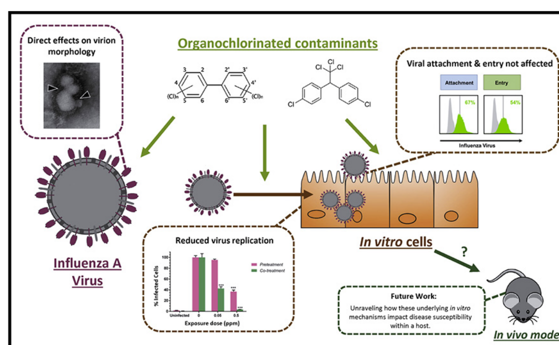
Jean-Pierre Desforges [a,*], Christopher Bandoro [b,c], Laila Shehata [c], Christian Sonne [a], Rune Dietz [a], Wendy B. Puryear [b], Jonathan A. Runstadler [b]

[a] *Department of Bioscience, Arctic Research Centre, Aarhus University, DK-4000 Roskilde, Denmark*
[b] *Department of Infectious Disease and Global Health, Cummings School of Veterinary Medicine at Tufts University, 200 Westboro Road, North Grafton, MA 01536, United States*
[c] *Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, United States*

HIGHLIGHTS

- Exposure to environmental contaminants reduces *in vitro* influenza infection.
- Effect is not mediated *via* reduced viral attachment and entry into exposed host cells.
- Exposure caused changes in virion surface morphology and viability.
- Mechanisms of contaminant effects on viral infections are complex.

GRAPHICAL ABSTRACT



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ABSTRACT

Environmental chemicals, particularly organochlorinated contaminants (OCs), are associated with a ranged of adverse health effects, including impairment of the immune system and antiviral immunity. Influenza A virus (IAV) is an infectious disease of major global public health concern and exposure to OCs can increase the susceptibility, morbidity, and mortality to disease. It is however unclear how pollutants are interacting and affecting the outcome of viral infections at the cellular level. In this study, we investigated the effects of a mixture of environmentally relevant OCs on IAV infectivity upon *in vitro* exposure in Madin Darby Canine Kidney (MDCK) cells and human lung epithelial cells (A549). Exposure to OCs reduced IAV infectivity in MDCK and A549 cells during both short (18–24 h) and long-term (72 h) infections at 0.05 and 0.5 ppm, and effects were more pronounced in cells co-treated with OCs and IAV than pre-treated with OCs prior to IAV ($p < 0.001$). Pre-treatment of host cells with OCs did not affect IAV cell surface attachment or entry. Visualization of IAV by transmission electron microscopy revealed increased envelope deformations and fewer intact virions during OC exposure. Taken together, our results suggest that disruption of IAV infection upon *in vitro* exposure to OCs was not due to host-cell effects influencing viral attachment and entry, but perhaps mediated by direct effects on viral particles or cellular processes involved in host-virus interactions. *In vitro* infectivity studies such as ours can shed light on the complex processes underlying host-pathogen-pollutant interactions.

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* Corresponding author at: Frederiksborgvej 399, Aarhus University, 4000 Roskilde, Denmark.
E-mail address: jpd@bios.au.dk. (J.-P. Desforges).

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1. Introduction

Host susceptibility to infectious disease is determined by factors associated with the host, the pathogen, and the environment (Lawrence and Vorderstrasse, 2013). Environmental contaminants are an important and often overlooked factor in disease outcomes and have been shown to contribute to the etiology and severity of various infectious and non-infectious diseases (Lawrence, 2007). For example, large population studies in Hong Kong and Brisbane have shown that air pollution is significantly associated with influenza A virus (IAV) hospitalizations, illness, and mortality (Wong et al., 2009; Xu et al., 2013). Furthermore, several cohort and case-studies of occupational, accidental release, and environmental exposure to contaminants, including organochlorinated contaminants (OCs) such as polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs), have documented altered immune response and disease and health outcomes in humans. Among many other outcomes, these studies have shown that pre and post-natal exposure to OCs in human populations is linked to increased incidence of respiratory and middle ear infections (Dallaire et al., 2006; Stølevik et al., 2011; Weisglas-Kuperus et al., 2004; Weisglas-kuperus et al., 2000), increased risk to infectious diseases like measles and meningitis (Carpenter, 2006; Dewailly et al., 2000), reduced anti-body responses to vaccinations (Grandjean et al., 2012; Heilmann et al., 2006), increased risk of asthma (Hansen et al., 2014), and increased risk of various cancers (Bonefeld-Jorgensen et al., 2011; Buckley et al., 1989; Turner et al., 2010).

Laboratory animal and wildlife studies support the link between pollutant exposure and susceptibility to disease. One of the most sensitive targets of the highly toxic OC dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)) is impaired host-resistance following exposure to IAV. Studies have shown that TCDD dose-dependently increases mortality in otherwise non-lethal IAV respiratory infections and alters cellular and humoral immunity, including both cellularity and function, as well as release of immune modulating cytokines (Burleson et al., 1996; Lawrence et al., 2000; Luebke et al., 2002; Vorderstrasse et al., 2003; Warren et al., 2000). The mechanism behind pollutant induced mortality in host-resistance models has been elusive over the past decades as various studies report often conflicting results. Yang et al. (1994) observed significantly higher IAV titers in lungs of TCDD treated rats (*Rattus* sp.), and similar increased viral replication after TCDD exposure has been shown after *in vitro* infections with human immunodeficiency virus 1 (HIV-1), Bovine Herpesvirus type-1 (BHV-1), and human cytomegalovirus (CMV) (Fiorito et al., 2008; Murayama et al., 2002; Tsyrlovts and Pokrovsky, 1993). In contrast, Lawrence et al. (2000) found reduced IAV loads in lungs of TCDD treated mice (*Mus musculus*), while Burleson et al. (1996) and Luebke et al. (2002) failed to find any association between TCDD-induced mortality and IAV titers. Whether differences between studies are due to experimental design or other factors remains unclear, nonetheless, there is ample evidence demonstrating the immunomodulatory and cellular effects of OCs in host tissues (Ilbäck and Friman, 2007; Lawrence, 2007; Luebke et al., 2002; Santoro et al., 2015; Vorderstrasse et al., 2003), ultimately highlighting the multiple and complex pathways through which contaminants may influence the host, virus, or host-virus interactions.

Influenza A virus is a major public health concern globally. Several devastating global pandemics of IAV have occurred over the past century including the Spanish Flu of 1918 which resulted in the death of an estimated 50 million people worldwide (Taubenberger and Morens, 2006). More recently, the 2009 pandemic H1N1 outbreak caused the death of at least 300 and infected an estimated one million people in the United States alone (Henderson et al., 2009). Mortality and morbidity during IAV infections is typically associated with pathology of the respiratory tract linked with secondary bacterial infections (Blevins et al., 2014; McNamee and Harmsen, 2006; Metzger and Sun, 2013). Given the global importance of IAV and documented effects of contaminants on disease susceptibility, there is serious need to better understand the interactive processes between host cells, virions, and contaminants.

The present study was designed to evaluate the effect of a mixture of environmentally relevant contaminants on IAV infectivity upon *in vitro* exposure. We use Madin-Darby Canine Kidney Epithelial (MDCK) and human Adenocarcinomic Alveolar Basal Epithelial cells (A549) in controlled *in vitro* models of IAV infectivity to assess how contaminants interact with both the host-cells and the virus. Our approach here of using a mixture of OCs is an important step in assessing the threat of more realistic exposomes for humans and wildlife, and sets up future studies to explore the mechanisms responsible for contaminant modulation of viral infections.

2. Materials and methods

2.1. Viruses and cell-lines

Viruses used in this study are H1N1 PR8 (A/Puerto Rico/8/1934) and a reverse-genetics engineered H1N1 WSN PB1flank-eGFP (obtained from the laboratory of Jesse Bloom), referred to in this study as H1N1 WSN-GFP, in which the PB1 gene's coding sequence was replaced by a gene encoding enhanced green fluorescent protein (eGFP) (Bloom et al., 2010). Viruses were propagated in either MDCK cells (obtained from American Type Culture Collection), human A549 cells (obtained from American Type Culture Collection), or MDCK-SIAT1-CMV-PB1 cells (obtained from the laboratory of Jesse Bloom) for 72 h. MDCK-SIAT1-CMV-PB1 cells are MDCK cells that overexpress α2,6-sialic acid and constitutively express the influenza polymerase PB1 protein (Bloom et al., 2010). This cell-line permits replication of the modified H1N1 WSN-GFP resulting in GFP expression in infected cells. The human H1N1 PR8 virus was further purified and concentrated through a 30% sucrose cushion during ultracentrifugation (Romanova et al., 2003).

MDCK cells and MDCK-SIAT1-CMV-PB1 cells were grown in DMEM (Hyclone) supplemented with 10% FBS (Seradigm), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma) at 37 °C with 5% CO_2. H1N1 PR8 infections were carried out in DMEM supplemented with 0.2% BSA (ThermoFisher), 1 μg/mL TPCK-trypsin (Sigma-Aldrich), 25 mM HEPES (Corning), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C with 5% CO_2. H1N1 WSN PB1flank-eGFP infections were carried out in MDCK-SIAT1-CMV-PB1 cells in OptiMEM (Hyclone) supplemented with 0.01% FBS, 0.3% BSA, 100 U/mL penicillin and 100 μg/mL streptomycin, and 100 μg/mL calcium chloride at 37 °C with 5% CO_2.

2.2. Virus titers and infectivity

H1N1 WSN PB1flank-eGFP was initially titered as described previously (Bloom et al., 2010). Briefly, serial dilutions of the stock virus were allowed to infect 1.0×10^5 MDCK-SIAT1-CMV-PB1 cells for 18 h at 37 °C with 5% CO_2. The % infected cells was determined by flow cytometry (Accuri C6, Accuri Cytometers), using uninfected control cells as the baseline and setting 0.5% of the control cells as GFP-positive (FlowJo). We then used the Poisson equation to calculate the number of infectious units (IU)/mL of the virus in initial stock. To compare samples across independent replicates, we normalized the % infected cells to that of the unexposed control (0.5% DMSO). H1N1 PR8 was titered in MDCK cells by tissue-culture infectious dose 50 (TCID50) assays (Balish et al., 2013). Briefly, 3.0×10^4 MDCK cells were seeded into 96-well plates (VWR) overnight and then viruses were serially diluted across the plate, incubated for 2 h to allow attachment, washed, and then returned to incubate for 72 h at 37 °C with 5% CO_2. Presence or absence of cytopathic effect (CPE) was observed and the TCID50/mL was calculated using the Reed-Muench method (Reed and Muench, 1938).

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