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Susceptibility of different cell lines to Avian and Swine Influenza viruses

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A B S T R A C T

Influenza outbreaks are widespread in swine and avian populations. Disease control is jeopardized by the extreme antigenic variability of virus strains. Primary isolation of Influenza virus is performed using embryonated chicken eggs (ECE), but alternatives to ECE are badly needed. Although various cultured cells have been used for propagating Influenza A viruses, few types of cells can efficiently support virus replication. One of the most commonly cell lines used in order to isolate Influenza A virus, is represented by the Madin Darby Canine Kidney (MDCK) cell line, but cells derived from primary swine organs (kidney, testicle, lung and trachea) can also be employed. The aim of this study was the evaluation of NSK, MDCK, UMNSAH/DF1 cell lines suitability, compared to ECE for isolation and propagation of Avian and Swine virus subtypes. The results indicated both NSK and MDCK could provide an appropriate substrate for cultivating either Avian (AIV) or Swine (SIV) Influenza virus strains, especially for high pathogenicity Avian Influenza ones.

Furthermore, NSK appeared more susceptible than MDCK cells for primary isolation of AIV. In contrast, UMNSAH/DF1 cell line seemed to be less permissive to support Avian virus growth. Furthermore, no SIV replication was detected except for one subtype. Additionally, the results of this study indicated that not all virus strains seemed to adapt with the same efficiency to the different cell lines. On the contrary, chicken embryos were shown to be the most suitable biological system for AIV isolation.

1. Introduction

Influenza A viruses are enveloped, single-stranded RNA viruses, which belong to the *Orthomyxoviridae* family. Based on the antigenicity of their surface glycoproteins represented by hemagglutinin and neuraminidase molecules, they are classified currently into 16 hemagglutinin (H1–H16) and 9 neuraminidase (N1–N9) subtypes (Fouchier et al., 2005). Their reassortment gives origin to several subtypes with varying pathogenicity degrees. High Pathogenicity Avian Influenza (HPAI) viruses evolve from low pathogenic precursors specifying HA subtypes H5 or H7; this process is characterized by the acquisition of a polybasic HA cleavage site. Functionally, virus infection of host cells is mediated by the binding between HA and sialic acid-containing receptors (Olsen et al., 2006). The HPAI form, in contrast to Low Pathogenic (LPAI) one, can be responsible for severe outbreaks with extraordinary mortality causing severe losses to the poultry industry. In contrast, there are fewer hemagglutinin and neuraminidase types circulating in mammals, particularly in pigs, in which H1N1, H3N2 and H1N2 are the three main currently widespread subtypes (Massin et al., 2010; Olsen et al., 2006). All the three viral subtypes (H1N1, H3N2, H1N2) have been associated with disease and there are no indications of differences in virulence among subtypes (Van Reeth, 2007). A common feature of influenza viruses from different species and subtypes, is their capacity to replicate in embryonated chicken eggs (ECE), that represent the gold standard for virus isolation and replication, also used for vaccine manufacturing (Yeolekar and Dhere, 2012).

ECE can support the growth of a broad range of Influenza viruses, although many field isolates do not readily grow. Also, availability of a sufficient number of high-quality ECE is a considerable limitation to their use. Furthermore, facts show that serial propagation of Influenza viruses in ECE can cause mutations in the hemagglutinin glycoprotein, leading to egg-adapted variants that may be antigenically different from the original field isolates and with variations in their glycosylation patterns (Schild et al., 1983; Audsley and Tannock, 2008). This may result in vaccine failure (poor inhibition of field virus replication and transmission by infected birds). In a scenario of a pandemic influenza outbreak, egg propagation system may not be adequate to face an emergency situation. In fact, it would be necessary to arrange a high number of vaccine doses in a limited period of time. For this reason, it seems to be useful to improve alternative diagnostic/productive tools, different from embryonated eggs (Liu et al., 2012).

Another important aspect to consider working with ECE, is represented by the limited flexibility for increased vaccine

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manufacture, the possible abrupt interruption of the supply chain due to disease in layer flocks, the risks concerning sterility during processing of infected allantoic fluids and the poor growth of some reassortant vaccine strains in embryonated eggs (George et al., 2010; Moresco et al., 2010).

At present, vaccine viruses are propagated in ECE, for this reason it would be important to consider the risk of egg-derived vaccines for individuals with allergies to ovalbumin and other egg proteins. Although the purification process is effective, the protein of the chicken embryo cannot be completely removed and may cause allergic reactions in vaccinated population.

In order to overcome these problems, many attempts have been made to use cell culture technology as a suitable alternative for influenza virus isolation and vaccine production on a large scale, as cell cultures can be manipulated easily with reduced time expenditure (Ferrari et al., 2003; Lee et al., 2008; Chiapponi et al., 2010).

In particular, use of continuous cell lines for influenza virus growth offers several advantages, including the opportunity to use fully characterized and standardized cells (Tree et al., 2001). Several types of primary, diploid and established cell lines are known to allow Influenza virus replication (Govorkova et al., 1996; Li et al., 2009); although the permissibility of most cell lines to Influenza virus infection is low and titres are lower than those detected by ECE (Hussain et al., 2010). Among them, Madin Darby Canine Kidney (MDCK) cell line was found to enable efficient viral growth and to support virus isolation from pathological specimens (Tobita et al., 1975; Meguro et al., 1979; Root et al., 1984; Voeten et al., 1999; Liu et al., 2009). Similar findings were obtained with African Green Monkey Kidney (VERO) cell line, which is now considered to be suitable for virus isolation from clinical specimens and growth of vaccine viruses (Kaverin et al., 1995; Govorkova et al., 1996; Kistner et al., 2007; Genzel et al., 2010).

Other cell lines were investigated for permissiveness to Avian Influenza viruses (AIV), such as Embryonic Swine Kidney (ESK), Hamster Lung (HmLu-1) and Monkey Kidney (JTC-12) (Sugimura et al., 2000). MDCK cell line has been the most consistently used for culturing and propagating AIV (Moresco et al., 2010).

Regarding Swine Influenza virus (SIV), chicken embryo is the most suitable biological method for virus isolation, on the other hand some studies have demonstrated the usefulness of different cell cultures as an alternative to ECE (Clavijo et al., 2002; Chiapponi et al., 2010). In these cases, authors underlined the possible link between viral hemagglutination and isolation rate on different substrates (MDCK, CACO-2 and ECE). In Chiapponi et al. (2010), it was studied the comparison among CACO-2, MDCK and ECE, in order to verify the real suitability of these biological tools during *in vitro* SIV isolation. The increased sensitivity of CACO-2 line than MDCK and ECE to isolate H1N1 and H1N2 subtypes. Otherwise, H3N2 virus detection was highest using ECE has been demonstrated. Clavijo et al. (2002) set forth the high variability of Swine Influenza virus; this aspect suggested, as the most efficacious diagnostic tool, the twin course use of both ECE and MDCK for the primary SIV isolation.

In the present study, mammalian and avian cell lines were compared for their ability to support either virus isolation from infected target specimens, or growth of AIV and SIV subtypes; the aim was the selection of several cell lines that could be used as alternative to ECE. Furthermore, the Intravenous Pathogenicity Index (IVPI) was performed in ECE and cell cultures at the first and tenth passage of each Avian virus subtype, in order to evaluate the changes that could occur during serial cultivation.

This first phase will be improved by further investigation regarding possible genomic virus changes during serial passages in cell cultures. This step is undertaken by genetic analysis.

2. Materials and methods

2.1. Cells

The selected cell lines were: Newborn Swine Kidney (NSK, BS CL 177), Madin Darby Canine Kidney (MDCK, BS CL 64) and Chicken Embryo Fibroblasts (UMNSAH/DF1, BS CL 197). These cell lines were registered in the cell culture bank of IZSLER, Brescia, Italy. Both NSK and MDCK were propagated in Minimal Essential Medium (E-MEM, Sigma–Aldrich, Milano, Italy) free of antibiotics and supplemented with 10% (v/v) foetal bovine serum (FBS, Mascia Brunelli S.p.A., Milano, Italy) and L-glutamine (4 mM/l, Sigma–Aldrich, Milano, Italy). UMNSAH/DF1 were cultivated in Dulbecco's modified Eagle medium (D-MEM, Sigma–Aldrich, Milano, Italy) supplemented with 10% (v/v) FBS. All cell lines were grown and maintained at 37 °C in 5% CO_2. Sub-passages were performed when cell monolayers reached confluence.

2.2. Viruses and virus titration

The following subtypes of AIV were selected: H5N1 LPAI A/Mallard/Italy/3401/05; H5N2 HPAI A/Chicken/Italy/8/A98; H7N1 HPAI A/Turkey/Italy/4580/99; H7N3 LPAI A/Turkey/Italy/2962/V03; they were field isolates. Finally, H9N2 LPAI A/Turkey Wisconsin/66 was a reference strain. All the samples were provided by Istituto Zooprofilattico Sperimentale delle Venezie, Padua, Italy.

SIV strains were: H1N1 A/Swine/Italy/25674/09, H1N1 A/Swine/Italy/186798/09, pandemic H1N1 A/Swine/Italy/290271/09, H1N2 A/Swine/BS2156, and H3N2 A/Swine/Oedenrode/1/96. They were field strains and provided by virus diagnosis laboratories (Parma and Brescia) of IZSLER.

All samples were inoculated in ECE at least 2 times in order to isolate the subtypes. Regarding H9N2 LPAI A/Turkey/Wisconsin/66, the previous passages were unknown.

Each virus was propagated in 10-day old, Specific Pathogen Free (SPF) embryos at 37 °C over 3 days. Allantoic fluids were collected after centrifugation at 1540 × *g* for 10 min and their hemagglutinating titres were evaluated using chicken red blood cells (RBCs) (0.5%) in phosphate buffer solution (PBS). The infectious titres were measured following inoculation of 100 μl of serial 10-fold dilutions (10^{-1} to 10^{-7}) into the allantoic cavity of three fertilized eggs. They were incubated for three days at 37 °C, the allantoic fluid was collected and virus growth was confirmed by a hemagglutination (HA) test. Embryo vitality was checked daily, in order to quickly verify the death, especially for HPAI strains. In case of death, the procedure was performed (as aforementioned) immediately. Infectious titres were calculated as described by Reed and Münch (1938) and expressed as Embryo Infectious Dose 50% (EID_{50}) per 0.1 ml.

2.3. Isolation of AIV from target tissues

Twenty, 6-week-old susceptible SPF White Leghorn chickens, subdivided into 4 groups of 5 animals each, were infected by the ocular-nasal (o.n.) route with 100 μl of the H5N1 LPAI A/Mallard/Italy/3401/05, H5N2 HPAI A/Chicken/Italy/8/A98; H7N1 HPAI A/Turkey/Italy/4580/99, and H7N3 LPAI A/Turkey/Italy/2962/V03 viruses, with a EID_{50} of 10^3/0.1 ml. These experimental infections were performed at Istituto Zooprofilattico, Padua, Italy. The infections were carried out in isolation facilities and all the procedures were performed according to the local guidelines on animal welfare and all groups were housed in cages set in isolators with water and food *ad libitum*.

Birds were observed daily for clinical signs. At the time of death, after H5N2 and H7N1 HPAI virus infections, target tissues (intestine,