Journal of Virological Methods 217 (2015) 55–63

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Optimization of an enzyme-linked lectin assay suitable for rapid antigenic characterization of the neuraminidase of human influenza A(H3N2) viruses

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Article history:
Received 4 October 2014
Received in revised form 6 February 2015
Accepted 16 February 2015
Available online 21 February 2015

Keywords:
Influenza
Neuraminidase
Antigenic drift
ELLA

A B S T R A C T

Antibodies to neuraminidase (NA), the second most abundant surface protein of the influenza virus, contribute to protection against influenza virus infection. Although traditional and miniaturized thiobarbituric acid (TBA) neuraminidase inhibition (NI) assays have been successfully used to characterize the antigenic properties of NA, these methods are cumbersome and not easily amendable to rapid screening. An additional difficulty of the NI assay is the interference by hemagglutinin (HA)-specific antibodies. To prevent interference of HA-specific antibodies, most NI assays are performed with recombinant viruses containing a mismatched HA. However, generation of these viruses is time consuming and unsuitable for large-scale surveillance. The feasibility of using the recently developed enzyme-linked lectin assay (ELLA) to evaluate the antigenic relatedness of NA of wild type A(H3N2) viruses was assessed. Rather than using recombinant viruses, wild type A(H3N2) viruses were used as antigen with ferret sera elicited against recombinant viruses with a mismatched HA. In this study, details of the critical steps that are needed to modify and optimize the NI ELLA in a format that is reproducible, highly sensitive, and useful for influenza virus surveillance to monitor antigenic drift of NA are provided.

1. Introduction

Influenza vaccines reduce the morbidity and mortality associated with annual influenza epidemics. The seasonal influenza vaccine is designed to protect against circulating influenza A H1N1 viruses (A(H1N1)), influenza A H3N2 viruses (A(H3N2)), and influenza B viruses. The influenza virus escapes host immunity through mutations in the major surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). This process is known as antigenic drift (Schild et al., 1974; Webster et al., 1982) and as a result of this drift, the influenza vaccine has to be updated frequently. In the period from 1999 to 2010, the A(H3N2) virus component was updated 6 times (Barr et al., 2010). Recently, it has been shown that only few mutations near the receptor-binding site of HA are responsible for antigenic drift of A(H3N2) viruses circulating between 1968 and 2003 (Koel et al., 2013). For NA, a number of antigenic sites have been described (Air et al., 1985). These antigenic regions surround the enzyme's active site (Colman et al., 1983, 1987) and are highly variable, most likely due to immune pressure (Laver et al., 1982; Luther et al., 1984). Influenza virus surveillance by national influenza centers is done year-round (Russell et al., 2008; Barr et al., 2010). Representatives of the predominant circulating viruses are sent to the World Health Organization (WHO) Collaborating Centers. These centers characterize the viruses by sequencing the HA and NA genes and performing hemagglutination inhibition (HI) assays (Barr et al., 2010). During vaccine strain selection, the main focus is on the genetic and antigenic characterization of HA (Fiore et al., 2010).

Influenza viruses attach to the host cell surface via binding of the HA to sialic acid-containing receptors (Sauter et al., 1989). The enzymatic activity of NA allows virus release from the cell (Palese et al., 1974; Palese and Compans, 1976; Liu et al., 1995) by cleaving the sialic acid residues from the newly formed virus particles and from the host cell (von Itzstein, 2007). Preclinical and clinical studies showed that NA-specific immunity can reduce the severity of disease (Schulman et al., 1968; Murphy et al., 1972; Couch et al.,

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http://dx.doi.org/10.1016/j.jviromet.2015.02.014
0166-0934/

1974; Kilbourne, 1976; Johansson et al., 1993; Brett and Johansson, 2005). Antibodies directed toward NA inhibit release and spread of newly formed virus particles from infected cells (Compans et al., 1969). The antigenic drift of NA does not closely correspond to that of HA (Schulman and Kilbourne, 1969; Kilbourne et al., 1990; Sandbulte et al., 2011). Considering these findings, investigating options to include routine analysis of NA during vaccine strain selection next to HA seems to be warranted.

Antigenic characterization of NA can be performed using NA inhibition (NI) assays to determine the extent of antibody-mediated interference with enzyme activity (Kilbourne et al., 1968). These assays rely on the enzymatic sialidase activity by measuring cleavage of sialic acid from highly glycosylated proteins such as fetuin. The NI thiobarbituric acid (TBA) assay (Warren, 1959; Webster and Laver, 1967) is based on the detection of free sialic acid. This assay is recommended by the WHO (Cox et al., 2002), but the use of large volumes of hazardous chemicals and laborious procedures has impeded antigenic characterization of NA during influenza virus surveillance. A simplified and miniaturized version of the TBA was developed (Sandbulte et al., 2009), but this assay still remains cumbersome. The recently developed enzyme-linked lectin assay (ELLA) (Lambre et al., 1991; Cate et al., 2010; Couch et al., 2012, 2013; Fritz et al., 2012; Couzens et al., 2014) also relies on the sialidase activity of NA, but instead of measuring free sialic acid, it detects the terminal galactose that becomes exposed after sialic acid cleavage by NA.

A complication of NI assays is the interference of HA-specific antibodies that block NA activity non-specifically when they bind to HA (Schulman and Kilbourne, 1969). Recombinant influenza viruses with a heterologous HA are commonly used for NI assays. Antibodies directed toward the H1 or H3 HA of A(H1N1) and A(H3N2) viruses do not cross-react with a heterologous HA (e.g. H6), and hence only the interaction between NA and NA-specific antibodies is measured (Couzens et al., 2014). However, the generation of recombinant viruses is time-consuming for large numbers of viruses and therefore this method is not suitable for large-scale surveillance of antigenic drift of circulating influenza viruses. For analysis of the antigenic drift of NA, it would be ideal to have the capability of using wild type viruses as antigen in assays that are not impacted by non-specific inhibitors, including antibodies to HA.

In this study, optimized methods to enable rapid antigenic characterization of NA, with wild type viruses as antigen, are described. Since the ELLA is less laborious and shows a good correlation to the miniaturized TBA (Fritz et al., 2012), this assay was selected as a platform. To prevent interference by antibodies directed against HA of wild type A(H3N2) viruses, ferret sera were raised against recombinant influenza A H7N2 viruses (A(H7N2)) viruses that contain the NA of various A(H3N2) viruses. Through this approach it is possible to screen wild type viruses, thus preventing the time-consuming generation of recombinant viruses or proteins for each virus of interest. Reproducibility and sensitivity of the NI assay were highest using virus concentrations that resulted in ~50% of total NA activity of that virus. Non-specific inhibition of ferret sera was observed for some wild type viruses, especially A(H3N2) viruses, but the critical steps to overcome this non-specific inhibition and obtain reproducible and highly sensitive results are also described.

2. Materials and methods

2.1. Cells

Madin–Darby canine kidney (MDCK) cells were cultured in Eagle's minimal essential medium (EMEM, Lonza, Breda, The Netherlands) supplemented with 10% fetal bovine serum (FBS, Sigma–Aldrich, St. Louis, MO, USA), 100 U/ml penicillin (Lonza), 100 U/ml streptomycin (Lonza), 2 mM glutamine (Lonza), 1.5 mg/ml sodium bicarbonate (Lonza), 10 mM HEPES (Lonza), and non-essential amino acids (MP Biomedicals, Europe, Illkirch, France).

293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate (Life Technologies, Bleiswijk, The Netherlands), 500 μg/ml geneticin (Life Technologies) and non-essential amino acids.

2.2. Plasmids

The NA gene segments of A(H3N2) viruses A/Bilthoven/21793/1972 (BI/72); A/Bilthoven/1761/1976 (BI/76); A/Netherlands/233/1982 (NL/82); A/Netherlands/823/1992 (NL/92); A/Netherlands/178/1995 (NL/95); A/Netherlands/69/2009 (NL/09) and the HA and NA gene segments of A(H2N2) viruses A/Netherlands/M1/1957 (NL/57) and A/Netherlands/B1/1968 (NL/68) were amplified by reverse transcription polymerase chain reaction (RT-PCR) and cloned in a modified version of the bidirectional reverse genetics plasmid pHW2000 (Hoffmann et al., 2000; de Wit et al., 2004). To reduce pathogenicity, the multibasic cleavage site (MBCS) was removed from the bidirectional reverse genetics HA plasmid of the highly pathogenic avian A(H7N7) virus (A/Netherlands/33/03) (de Wit et al., 2010). This was done using the QuikChange multi-site-directed mutagenesis kit (Stratagene, Huissen, The Netherlands) according to the instructions of the manufacturer with specific primers (available from the authors upon request). The plasmids encoding the internal genes of A/Netherlands/219/03 (H7N7) have been described previously (de Wit et al., 2010).

The accession numbers that were used are as follows: for NA, CY112307, CY113199, CY114439, CY113735, CY116590, CY113023, KM402803, and KM402811; for HA, KM402801 and KM402809.

2.3. Viruses

All human and recombinant influenza A viruses were propagated at 37 °C and influenza B viruses at 33 °C in MDCK cells in EMEM supplemented with, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 1.5 mg/mlsodium bicarbonate, 10 mM HEPES, non-essential amino acids and 1 μg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma–Aldrich, Zwijndrecht, The Netherlands). Avian influenza viruses were propagated in 11-day old embryonated chicken eggs. All viruses were harvested 48 h post-inoculation and cell debris was removed by centrifugation for 15 min at 3000 rpm. Supernatant was either immediately stored in suitable aliquots at -80 °C or, if needed, after concentration using an Amicon Ultra-15 Centrifugal Filter (Millipore, Amsterdam, The Netherlands).

Recombinant A(H2N2) and A(H7N2) viruses were generated by reverse genetics using transient calcium phosphate-mediated transfections of 293T cells as described previously (de Wit et al., 2004). A(H2N2) viruses were generated with plasmids carrying the HA and NA gene segments of A(H2N2) viruses (NL/57 or NL/68) and the six remaining gene segments of A/Puerto Rico/8/1934 (H1N1) under biosafety level 2 (BSL-2) conditions. A(H7N2) viruses were generated under biosafety level 3 (BSL-3) conditions with plasmids carrying the HA gene segment of A/Netherlands/33/03 (H7N7) without the MBCS, the internal gene segments of A/Netherlands/219/03 (H7N7) (de Wit et al., 2010), and one of the following NA gene segments of A(H3N2) viruses: A/Bilthoven/16190/1968 (BI/68) (Schrauwen et al., 2011); BI/72; BI/76; NL/82; NL/92; NL/95; A/Netherlands/213/2003 (NL/03) (Chutinimitkul et al., 2010); NL/09; and A(H2N2) viruses: NL/57 or NL/68. The supernatant of the transfected cells was harvested 48 h