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A novel method for detection of H9N2 influenza viruses by an aptamer-real time-PCR

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A B S T R A C T

H9N2 Influenza subtype has emerged in Tunisia causing epidemics in poultry and resulting in major economic losses. New mutations in their hemagglutinin and neuraminidase proteins were acquired, suggesting their potential to directly infect humans. Effective surveillance tools should be implemented to help prevent potential spillover of the virus across species. We have developed a highly sensitive real time immuno-polymerase chain reaction (RT-I-PCR) method for detecting H9N2 virus. The assay applies aptamers as ligands to capture and detect the virus. First, a panel of specific ssDNA aptamers was selected via a one step high stringency protocol. Next, the panel of selected aptamers was characterized for their affinities and their specificity to H9N2 virus. The aptamer showing the highest binding affinity to the virus was used as ligand to develop a highly sensitive sandwich Aptamer I-PCR. A 3-log increase in analytical sensitivity was achieved as compared to a routinely used ELISA antigen test, highlighting the potential of this approach to detect very low levels of virus particles. The test was validated using clinical samples and constitutes a rapid and a label-free platform, opening a new venue for the development of aptamer –based viability sensing for a variety of microorganisms of economic importance in Tunisia and surrounding regions.

1. Introduction

The poultry sector in Tunisia is highly developed and plays a significant role in the economic and the social environment of the country. This sector is threatened by zoonotic diseases that can result in significant losses to livestock and endanger human health. One such disease is the influenza virus H9N2, which has caused epidemics in several species and across several countries. The virus first emerged in Tunisia in 2009 and was responsible for several outbreaks in poultry, resulting in significant economic losses. Although H9N2 commonly infects poultry, strains isolated and characterized from Tunisian outbreaks in 2011, have shown a mutation in the viral hemagglutinin (HA) surface protein that may allow the virus to preferentially bind to human sialic receptor α (2, 6) (Tombari et al., 2011). These mutations suggest the potential of H9N2 virus to become highly pathogenic, thus posing a threat to both the poultry sector and the human safety and health.

The emergence of such strains highlights the risks associated with current detection, diagnostic, and therapeutic techniques. To prevent serious outbreaks in both poultry and human population, continuous monitoring of viral genetic changes throughout the year and strict enforcement of control process and biosecurity measures are warranted. However, there is a lack of cost-effective, non-culture based, quick detection assays and effective therapeutics for these viruses. Current diagnostic methods for influenza are culture-based, relying on enrichment and antibodies for virus detection. Usually, these methods are based on antibody test kits and strip tests (Cho et al., 2013; Dusek et al., 2011), and antibody instability under field conditions limits application of these assays. Additionally, these methods are time consuming and often unreliable, making these approaches less than ideal for outbreak investigations and disease monitoring. To overcome these limita-

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tions, we are proposing the use of DNA aptamers as highly sensitive diagnostic tools and potential therapeutics.

DNA aptamers are functional short chain nucleic acids ranging of from 20 to 90 bases and their secondary structures offer a large area to recognize antigen, thus allowing them to bind a variety of molecules, ions, or whole cells. Their specific recognition of targets and their high affinity to nano or subnanomolar (Kikuchi et al., 2009), make them very powerful tools as bio-recognition ligands. Since the discovery of DNA aptamers, many applications have been widely extended from diagnostics to therapeutics, and used for biosensors development or specific delivery of active molecules (Dausse et al., 2009; Ozalp et al., 2011; Gopinath et al., 2012).

Due to their chemical properties, they are often compared to antibodies with desirable and interesting properties. They have low immunogenicity and high physical stability (Chelliserrykattil and Ellington, 2004; Kato et al., 2005); their production is largely economic and reproducible, and they are chemically derivable and controllable. Their major advantage is the ease of *in vitro* selection by Systematic Evolution of Ligands by EXponential enrichment (SELEX) from a pool of sequences generated randomly (ranging up to 10^{15} sequences). This is a robust combinatorial chemical screening method that can be performed quickly and completely *in vitro*. The selection process may extend between two to four weeks, which is significantly short in comparison to the generation of antibodies that needs months.

Currently, several examples have been reported for aptamers as diagnostic tools (Dausse et al., 2009; Song et al., 2008). Such diagnostic applications range from simple enzyme-linked immunosorbent assays (ELISA) to more complex ones with higher sensitivity (electrochemical, optical and sensitive analytical mass techniques) (Labib et al., 2012a, 2012b, 2012c; Zhou et al., 2010). These high affinity DNA or RNA aptamers have been successfully selected against viral proteins or whole virus (Chen et al., 2009; Feng et al., 2011; Konopka et al., 2000; Labib et al., 2012d). Furthermore, these aptamers were directed selected against hemagglutinin of avian influenza virus and were capable to prevent viral infection of the host (Cheng et al., 2008; Choi et al., 2011; Jeon et al., 2004; Park et al., 2011; Wongphatcharachai et al., 2013). Unlike antibodies, these aptamers were also able to distinguish between closely selected viral strains (Wongphatcharachai et al., 2013).

One such high sensitive assay is immuno-PCR technique. This hybrid test is based on immuno polymerase chain reaction (immuno-PCR) and enzyme-linked immunosorbent assay (ELISA) techniques. It is a very sensitive antigen detection system developed by Sano et al. in 1992) (Sano et al., 1992) and has been successfully used for the detection of small amounts of wide range of antigens like viral antigens (Barletta et al., 2009), bacterial antigens (Liang et al., 2003), prions (Gofflot et al., 2005), and bacterial toxins (Malou and Raoult, 2011; Zhang et al., 2008). The I-PCR uses a reporter DNA and a PCR for amplification instead of an enzyme (alkaline phosphatase (AP) or horseradish peroxidase) that catalyzes the substrat in ELISA test. The sensitivity of I-PCR is increased three to five logs (Adler et al., 2003; Barletta et al., 2004; Chao et al., 2004; Liang et al., 2003; Mweene et al., 1996; Saito et al., 1999) and known as one of the most sensitive detection method that exist (Malou and Raoult, 2011). The combination of an I-PCR approach and aptamers as ligand for the detection of Tunisian H9N2 virus is an original approach and has the potential to alleviate a number of issues posed by viral disease diagnostics and detection procedure limits. Such approach was tested and has improved 20000 fold the detection sensitivity, highlighting its potential to detect very low levels of target analytes (Pinto et al., 2009). Yoshida et al. (2009) developed sensitive detection system based on immuno-aptamers PCR using an RNA aptamer for rabbit IgG instead of the conventional secondary antibody. The same author summarized in his review the recent advances in the development of aptamer-based detection systems especially in their use in the amplification exponentially by PCR (Yoshida et al., 2012). Other team used this strategy to detect Fc fragments of mouse IgG (Liao et al., 2010). More recent colorimetric approach was used to detect NF-κB p50 by conversion of the presence of antigen in the reporter oligonucleotides through protein-DNA interaction, exonuclease III digestion, and isothermal exponential amplification (Zhang et al., 2012).

These assays show an extremely high sensitivity and a lowered limit of detection and can detect and quantify the presence of virus in swab samples, eliminating the need for culture-based approaches. This approach was applied in our study and could pave the development of an efficient aptamer for the diagnosis of virus of interest.

In this work, we selected and characterized DNA aptamers that specifically bind to the Tunisian H9N2 AIV, using a modified single round of selection protocol of SELEX. The best aptamer candidate was used to develop a highly sensitive assay that detects specifically the virus in a complex medium based on aptamer I-PCR assay.

2. Materials and methods

2.1. Virus strains and clinical samples

The avian influenza isolate A/CK/TUN/145/12 (H9N2), accession numbers KP058446 and KP058447 for HA and NA genes, respectively (unpublished), was used in the present study. Both H9N2 avian influenza isolates, A/Ck/TUN/12/10 and A/Migratory Bird/TUN/51/10, previously characterized by our group, were also used (Tombari et al., 2011). Live H120 vaccine strain of avian bronchitis virus and LaSota vaccine strain of Newcastle Disease were purchased from a local distributor. Infectious influenza virus B/Brisbane/60/2008 (NIBSC code: 12/200), A/Switzerland/9715293/2013 (H3N2) (NIBSC code: 14/224) and A/PR/8/34 (H1N1) (ATCC VR95) strains were kindly provided by Dr. Samia Rourou (Institut Pasteur de Tunis). Clinical samples from suspected poultry, received for laboratory diagnostic, were tracheal (ET) and cloacal (EC) swabs and internal organs (tonsils (TN), livers (L), spleens (S) lungs (LG) and kidneys (K)).

2.2. Production and purification of H9N2 virus

Isolated H9N2 virus was propagated in specific pathogen free (SPF) embryonated eggs chicken via the allantoic route. The eggs were incubated for 72–96 h at 37.5 °C. The collected allantoic fluids was added onto 1/3 vol of 30% sucrose and loaded for ultracentrifugation at 40000 rpm/min, for 2 h at 4 °C. The supernatant was discarded and the virus pellet was resuspended in PBS and conserved at −80 °C. An aliquot of these precipitated viruses was tittered using Vero cell cultures.

2.3. Single round selection of aptamers from ssDNA library

Aptamer candidates against H9N2 virus were selected using one step SELEX protocol. The protocol of one step selection was performed according to the publication of Arnold et al. (2012) (Arnold et al., 2012) with few modifications. A single-stranded aptamer library (WAP40m) (Lamont et al., 2014), consisting of a 40-mer randomized regions sequence flanked by constant-primer binding regions, was used (Integrated DNA Technologies, Inc., Coralville, IA). The elution of binding aptamer was performed using a gradient salt (NaCl) elution. The experiment was started by an immobilization of the virus to the 96-well ELISA plate. The well was dry blotted and 100 μL of single-stranded aptamer library (10 μM) was added and incubated for 1 h. After draying the plate, 100 μL of PBS was added, incubated for 5 min, then the solution collected, labeled, and