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Economic high-throughput-identification of influenza A subtypes from clinical specimens with a DNA-oligonucleotide microarray in an outbreak situation

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ABSTRACT

Influenza A surface proteins H (haemagglutinin) and N (neuraminidase) occur in sixteen and nine distinct genotypes, respectively. The need for a timely production of vaccinations in case of pandemics or seasonal epidemics requires rapid typing methods for the determination of these alleles. The aim of the present study was to develop and improve a rapid and economic assay for determining H and N subtypes of influenza A from patient samples. The assay is based on the hybridisation of labelled amplicons from H and N reverse transcriptase-PCRs using consensus primer pairs to subtype-specific probes on microtiterstripe-mounted DNA-microarrays. An algorithm for semi-automatic data interpretation of raw data and assignment to H and N subtypes was proposed. Altogether, 191 samples were genotyped. This included 134 patient and 44 reference samples as well as controls. Under routine conditions sensitivity and specificity proved to be comparable to conventional nested or real-time PCRs. At least 130 out of 147 array-positive samples were unambiguously assignable. This included all sixteen variants of H as well as all nine variants of N. Furthermore, eighty-two samples from the 2009/2010 “novel H1N1/swine flu” (SF)-outbreak were correctly identified.

1. Introduction

Air-borne infections such as influenza A are able to spread rapidly in a susceptible population, and they have the potential to cause pandemics. The influenza A virus belongs to the orthomyxoviridae [1]. It has a segmented genome of eight antisense RNA strands. The most important surface proteins are haemagglutinin (H), for which sixteen subtypes are currently known, and neuraminidase (N) with nine described subtypes. In case of a simultaneous infection of a host cell with multiple strains of influenza A, a recombination of genome segments can occur (antigenic shift). Additionally, sequences of genes and, consequently, antigenic properties of surface proteins might alter because of random mutations (antigenic drift). Due to this variability, immunity after influenza infections or vaccinations is strictly strain-specific.

The influenza A virus is able to infect different host species. The greatest diversity of subtypes can be found in wildfowl, but several mammals including pigs, horses and humans can also be infected. Because of host-specific polymorphisms in influenza viruses [2], infections of humans with animal strains are relatively rare. However, several large pandemics have been caused by recombinant virus strains that have evolved from parent strains adapted to humans, or other mammals such as swine, and birds [3]. For decades, H1N1 and H3N2 strains predominated [4]. However, recent outbreaks of H5N1 since 1997 and of a new H1N1 variant (“swine flu”, “Mexican flu” or “novel flu") since 2009 show that there is a permanent possibility of the emergence of new strains with new epidemiological properties.

The risk of a recombination event depends on the extent of co-circulation of different subtypes in a susceptible target population. For this reason, large-scale genotyping of influenza A viruses and continuous surveillance of humans and animals would help to assess and understand future risks. Additionally, developing new methods for typing high numbers of clinical specimen will overcome the difficulty of identifying an emerging strain in the early stage of an epidemic. Generic influenza assays are not able to

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distinguish emerging strains from the previously predominant strains and even miss new variants. Case numbers in this period will be low such that no specialised laboratory would pick up such an isolate.

In a pandemic situation, it can be crucial to obtain data on recent variation and recombination since this might affect the virulence, the susceptibility towards stockpiled neuraminidase inhibitors, and the efficiency of the current vaccination. In a defined epidemic or pandemic situation it is a reasonable strategy to combine a reverse transcriptase PCR (RT-PCR) for the detection of any influenza A strain with a subtype-specific assay such as the detection of H1. A more comprehensive characterisation of a given strain requires sequencing of several genes and/or cell culture assays. This usually is beyond the possibilities of a clinical laboratory, and thus it is restricted to few specialised scientific or reference laboratories.

Recent developments of the DNA-microarray technology can be used for large-scale typing of clinical influenza A specimens since comparatively inexpensive high-throughput systems have become available [5–10]. In the wake of the avian influenza (H5N1) outbreak, two arrays for typing of haemagglutinin and neuraminidase have been developed and tested [11,12]. These two arrays now have been combined. New primers and probes have been introduced in order to detect “swine flu”, *i.e.*, the novel H1N1 strain. Finally, the assay was transferred to a microtiterstripe format allowing simultaneous testing of eight up to 96 samples. In the present study, this technology has been evaluated using clinical samples from the University Hospital Dresden which have been collected during the seasonal influenza 2008/2009 and “novel H1N1/swine flu” outbreaks 2009.

2. Materials and methods

2.1. Samples

The Friedrich Loeffler Institute (FLI), Greifswald-Insel Riems, Germany, (which is the German National and OIE reference laboratory for avian influenza) provided twenty-two reference samples obtained from egg cultures. Three samples belonged to H1N1 (including one “novel H1N1/swine flu”), two to H3N2, and one to each of the following subtypes: H1N2, H2N3, H4N6, H5N1, H5N3, H6N2, H7N1, H7N7, H8N4, H9N2, H10N8, H11N9, H12N5, H13N6, H14N5, H15N9 and H16N3.

Twenty-two reference samples were obtained from INSTAND e.V., a German non-profit organisation overseeing quality control schemes in diagnostic laboratories and providing samples for quality control purposes. These samples originated from egg or cell cultures and some of them were chemically inactivated (see supplemental file for further details).

Twenty-two patient samples from cases with seasonal influenza were obtained from the University Hospital in Dresden, from its outpatient departments and from some collaborating laboratories and private practises. They were collected in 2009. All samples have been tested previously for influenza A by PCR using methods described below, but no further typing methods were used to determine subtype affiliations.

Eighty-two samples were obtained from patients with “novel H1N1/swine flu” during the 2009 outbreak. They originated from the same region as the seasonal influenza samples and were diagnosed as described below.

Beside these influenza A positive samples, 30 influenza A negative samples were included into the present study as controls. They were collected between March 2008 and December 2009. Ten of them were previously tested positive for influenza B. Another twenty were PCR-negative for both viruses, influenza A as well as B. In addition to the samples, in every experiment a negative control with PCR grade water was included and subsequently hybridised.

2.2. Conventional diagnostics of influenza A

All diagnostic samples were subjected to PCR assays. Three different systems were alternatively used. One was a nested PCR [13]. The other one was the commercial real-time system distributed by Artus (Hamburg, Germany). During the peak of a “novel H1N1/swine flu” outbreak, an H1-SF-specific real-time PCR was performed as previously described [14]. Urgent diagnostic samples were tested using the antigen detecting lateral flow assay BinaxNow® Influenza A & B (Alere GmbH, Cologne, Germany), but all of these samples were re-tested using PCR.

Influenza B was diagnosed using a nested PCR according to a published protocol [13].

2.3. Array design and procedures

The complete array is commercially available (FluType by Alere Technologies GmbH, Jena, Germany; http://identibac.com/en/assay-principle-products/products-available/influenza-genotyping.html). Amplification, labelling and hybridisation as well as analysis were performed according to the instructions of the manufacturer. Primers and probes have been previously described [11,12]. Each H allele was targeted by two (H14) to fifteen (H5) probes. For N alleles three (N6) to twenty-seven (N1) probes were included. Layout, primer and probe sequences are available on request.

In addition, new probes and new primers were introduced in order to detect “swine flu”, *i.e.*, novel H1N1. These were derived from the new H1N1 variant, GenBank entries FJ981615, GQ117032, GQ117119 *etc.*

In short, RNA from clinical samples (throat swabs, bronchoalveolar lavage *etc.*) was prepared using the QIAamp viral RNA mini Kit from Qiagen (Hilden, Germany) according to the protocol as supplied by the manufacturer (although using a re-elution volume of 100 µl). For RT-PCR, reagents from Superscript™ III One-Step RT-PCR by Invitrogen (Invitrogen, Carlsbad, USA), 2 µl sample RNA preparation and a mix of, respectively, H or N specific, 5′-biotin labelled primers were used. Reactions for H and N were performed separately. Amplification was performed using following program steps: pre-amplification (30 min at 50 °C and 2 min 94 °C), followed by 45 cycles (30 s at 94 °C; 45 s at 50 °C; 45 s at 68 °C) and a final elongation step (5 min at 68 °C). Resulting H amplicons were approximately 170 bp long (ranging from 164 bp to 176 bp, depending on virus type) while N amplicons had a length of *ca.* 650 bp.

Amplicons were used directly or stored overnight at 4–8 °C or, in case of longer storage periods, at −20 °C. Amplicons were diluted in a hybridisation buffer, denaturated for 5 min at 95 °C, shortly (1 min) cooled on ice, and hybridised to the array (60 min at 40 °C on a shaker). After washing steps, hybridisations were visualised by adding streptavidine-horseradish-peroxidase and by a subsequent peroxidase-triggered dye precipitation (HybKit Alere Technologies, Jena). This lead to the formation of visible spots on the array surface, which then were scanned, measured and analysed using a dedicated reader and software (ArrayMate Reader, Alere Technologies GmbH).

2.4. Algorithm for the interpretation of array data

The local backgrounds as well as spot intensities were measured, for the latter using only valid pixels within the automatically recognised spot area. Normalised intensities of the spot value were calculated according to the following equation: