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# Differentiation of Influenza B virus lineages Yamagata and Victoria by real-time PCR

**Running title: Influenza B Yamagata / Victoria real-time PCR**

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List of abbreviations

| | |
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| PCR | Polymerase Chain Reaction |
| HIT | Hemagglutination Inhibition Test |
| MGB | Minor Groove Binder |
| HA | Hemagglutination |

**Abstract**

Since the 1970ies, *influenza B* viruses diverge into two antigenically distinct virus lineages called Yamagata- and Victoria-lineage. We present the first real-time PCR assay for virus lineage discrimination to supplement the classical antigenic analyses. The assay was successfully applied to 310 primary samples collected in Germany from 2007 to 2009.

The *influenza* viruses are members of the *Orthomyxoviridae* family and are divided into three genera *A*, *B* and *C* (8). *Influenza A* and *B* viruses clinically are most relevant, since they cause severe respiratory infections in humans (2). While *influenza A* viruses comprise a large group of different subtypes (8), *influenza B* viruses used to form a homogenous group and only started to diverge into two antigenically distinguishable lineages in the 1970ies (3, 4, 6). These virus lineages were named after their first representatives, B/Victoria/2/87 and B/Yamagata/16/88, as Victoria- and Yamagata-lineage (6). Today, the antigenic differences between the lineages allow their discrimination by hemagglutination inhibition testing (HIT) using specific immune sera raised against contemporary strains of either lineage. However, HIT is a time-consuming and tedious process and needs virus isolation as a prerequisite. In contrast, PCR is well known to be a fast, specific and sensitive diagnostic method, and real-time PCR furthermore reduces the risk of carryover contamination and allows large-scale diagnostics (5). However, to date, no real-time PCR assay has been described which enables the differentiation of *influenza B* viruses, which would greatly speed up and thus improve *influenza* virus surveillance. We therefore present an assay which not only amplifies both lineages, but also discriminates between them by the application of two differently labelled minor groove binder (MGB) probes, either one being specific for one lineage.

The target region of the assay was chosen from an alignment with recent *influenza B* hemagglutinine database sequences (years 2000 – 2008). The 81bp amplicon comprises a 13bp stretch that differs in 6 positions between the two lineages. The stability of the characteristic nucleotide changes was confirmed by an alignment comprising all available *influenza B* hemagglutinine database sequences (1622 sequences, years 1954 – 2008). The distinctive nucleotides have been stable from the late 1990ies until today, so that nucleotide changes are not impossible, but

unlikely to occur within the near future. Thus, an MGB probe was designed for either lineage targeting this 13bp stretch. By the application of both probes with different colour labels (FAM and VIC) in a single PCR reaction, both virus lineages can be detected and discriminated simultaneously, as only one of the two probes will give a fluorescence signal.

Reaction conditions were established for the LightCycler 480 system in a total reaction volume of 25µL containing 1x PCR Buffer, 5mM $MgCl_2$, 1.25µM dNTP (Invitrogen, USA) with dUTP (GE Healthcare, Great Britain), 0.5U Platinum Taq polymerase (Invitrogen, USA), 900nM of forward primer F432 (5'-ACCCTACARAMTTggAACYTCAgg -3'), 600nM of reverse primer R479 (5'-ACAgCCCAAgCCATTgTTg -3'), 150nM of Yamagata probe MGB437 (5'- 6FAM – AATCCgMTYTTACTggTAg – MGB -3'), 100nM of Victoria probe MGB470 (5'- VIC – ATCCgTTTCCATTggTAA – MGB -3') and 3µL of template cDNA. Cycling conditions were 5min at 95°C, followed by 45 cycles of 15s at 95°C and 30s at 60°C.

The assay was evaluated using two plasmids that were cloned according to routine procedures (1) and contained 610/613bp of the hemagglutinine gene of B/Bayern/7/08 (plasmid pYam) and B/Berlin/38/08 (plasmid pVic), two contemporary German isolates representing the Yamagata and Victoria lineage. Thus, the complete primer- and probe-binding regions represent the original sequences of these two isolates. Amplification of 10-fold serial dilutions of each plasmid in λ-DNA (1ng/µL) revealed a linear detection range from $10^7$ to $10^2$ genome equivalents per reaction with a correlation ($R^2$) of >0.998 and a slope of -3.32 (pYam) and -3.33 (pVic) (Figure 1A), resembling a PCR efficiency of 1 ($E=10^{-1/slope} - 1$). We performed a probit analysis as a model of nonlinear regression that indicated a 95% detection probability of 24.4 genome equivalents per reaction for the pYam plasmid and 12.4 genome equivalents per reaction for pVic (Figure 1B). Additionally, from virus culture material

of the corresponding virus isolates B/Bayern/7/08 (Yamagata) and B/Berlin/38/08 (Victoria) the 95% detection probability was determined to be 1.3x $10^{-5}$ and 3.8x $10^{-5}$ HA units per reaction. The overall variability was assessed by repeat examination of three different plasmid copy numbers as well as virus culture material with a high, medium or low virus load. The standard deviations of $C_t$ values were found to be very low and were comparable for Yamagata and Victoria viruses and plasmids (Table 1). We found no cross-reactivity with DNA/cDNA of isolates from seasonal *influenza A* virus subtypes *H1N1* and *H3N2*, pandemic *influenza A/H1N1*, *Respiratory Syncytial Virus A* and *B*, *Adenovirus* serotypes 2, 3 and 4, *Human Metapneumovirus*, *Parainfluenza viruses 1*, *2* and *3*, *Coxsackievirus* and *Rhinovirus* as well as human DNA from swab samples.

Finally, to confirm the applicability of the assay in clinical diagnostics, we examined 310 *influenza B* virus-positive primary samples from the influenza seasons 2007/08 and 2008/09. All samples were taken from German patients presenting with influenza-like illness and successfully underwent HIT after virus isolation on MDCK2 cells. The nasal and throat swabs were washed in MEM cell culture medium immediately after arrival. RNA was extracted using either the RTP DNA/RNA Virus Mini Kit (Invitek) or the MagAttract Viral RNA M48 Kit (Qiagen) according to the manufacturer's suggestions. cDNA was synthesized from 25µL RNA applying the M-MLV Reverse Transcriptase (Invitrogen) and random hexamer primers as described elsewhere (7). Residual RNA was stored at -80°C until further use.

Applying the presented assay, viruses were amplified from all 310 primary samples with $C_t$ values between 22 and 37. All samples were genetically identified as Yamagata- or Victoria-lineage viruses in concordance with HIT results. The 310 primary samples comprised 185 Yamagata and 3 Victoria lineage viruses from the season 2007/08 as well as 120 Victoria and 2 Yamagata lineage viruses from the

season 2008/09. Since the assay's introduction into our diagnostic routine in February 2009, it has been run on approximately 5000 samples, and to our knowledge no false-positive or false-negative results have been obtained.

In summary, we present the first real-time PCR assay for the differentiation of *influenza B* viruses. This assay considerably speeds up virus lineage identification in clinical specimens and therefore will help to improve the surveillance of *influenza B* viruses. Furthermore, it will enable a timely recognition of the circulating B virus lineage during influenza seasons and thus will allow short-term decisions on patient care, e.g. in case of a non-matching vaccine, as well as the early onset of on-time epidemiological examinations, including WHO decisions on vaccine composition.

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**Figure Legends**

Table 1 PCR assay validation: Detection variability

Variability runs were performed by examination of pYam and pVic plasmid dilutions (5x $10^5$, 5x $10^3$, 5x $10^1$ genome equivalents per reaction) as well as on cultured virus material with high (6.67x $10^8$ genome copies/ml), medium (6.67x $10^6$ genome copies/ml) or low virus load (6.67x $10^4$ genome copies/ml). Intraassay variability was tested in sextuplicate reactions. Interassay variability was determined by twofold examination of duplicate reactions under inclusion of data from the intraassay variability run (total: threefold examination). The standard deviations (SD) of obtained $C_t$ values are listed.

Figure 1 PCR assay validation

(A) Mean $C_t$ values (double reactions) of plasmid dilutions containing $10^7$ – $10^2$ genome equivalents of pYam and pVic were plotted against the cycle number. Slope and correlation ($R^2$) are indicated.

(B) Probit analyses were performed by examination of plasmid dilutions containing 100 – 0.1 genome equivalents of pYam and pVic in tenfold reactions. Results were analyzed using the SPSS 17.0 statistics software.