Use of allele specific RT-qPCR to measure the differential infectivity of HA variants in human infection models using A/Wisconsin/67/2005.

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Background

The A/Wisconsin/67/2005 strain has been used extensively in human viral challenge models (hVCM). The HA1 coding region of A/Wisconsin/67/2005 has a natural single nucleotide polymorphism (SNP) at position 156, generating a 50:50 mixture of HA containing either a histidine (HA-H156) or glutamine (HA-Q156) at this position. This SNP is known to contribute to antigenic drift and be susceptible to egg-adapted mutations contributing to a marked decrease in vaccine effectiveness during the 2012-2013 season. Due to the proximity of the SNP to the receptor binding region in the globular head of HA, it is of interest to monitor any potential differences in infectivity between the HA-H172 and the HA-Q172 variant strains within the A/Wisconsin inoculum using the human viral challenge model (hVCM). We have designed Taqman probes enabling 100% discrimination between HA-H172 and HA-Q172 as a first step to analyzing the distribution of the SNP during late stages of infection. This approach allows extremely rapid quantitation of SNP (assay turnaround time of 1 day) when compared to deep sequencing techniques.

Introduction

A/Wisconsin/67/2005 grown in VERO cells has a SNP in HA1 at position 172 in HA1. This corresponds to amino acid 156 in the mature HA1 protein.

Figure 1. The HA sequence of an in-house A/Wisconsin/67/2005 strain is aligned with two reference HA sequences from GenBank using the AlignX program. Only a partial comparison is shown.

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(151)                151       160       170       180       190
A/Wisconsin/67/2005(143) TSSSCKRRSNNSFFSRLNWLTXLKFKYPALNVTMPNNEKFDKLYIW
EU103823 HA(151)         TSSSCKRRSNNSFFSRLNWLTHLKFKYPALNVTMPNNEKFDKLYIW
CY034116 HA(151)         TSSSCKRRSNNSFFSRLNWLTQLKFKYPALNVTMPNNEKFDKLYIW
Consensus(151) TSSSCKRRSNNSFFSRLNWLT LKFKYPALNVTMPNNEKFDKLYIW
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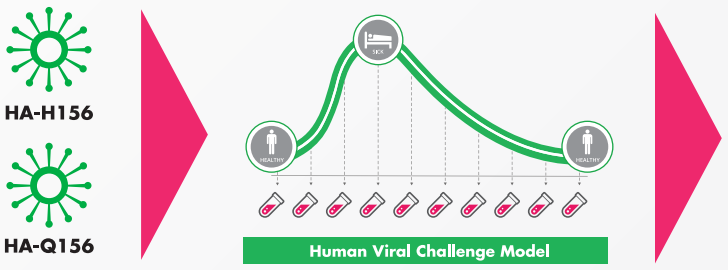
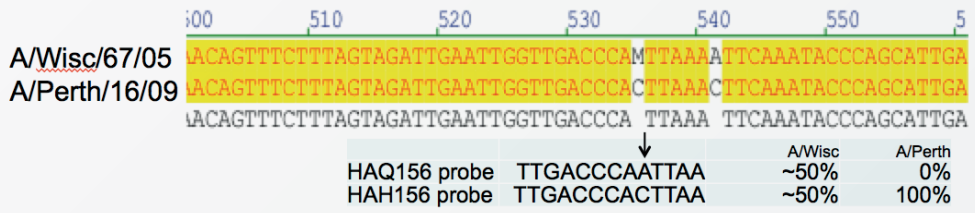
Sequence analysis of HA shows the SNP is present at a 50:50 ratio. A 5log TCID50/mL inoculum of virus was prepared for use in the hVCM.



Optimisation of allelic qPCR reagents provide 100% discrimination between HA-H156 and HA-Q156 variant sequences.

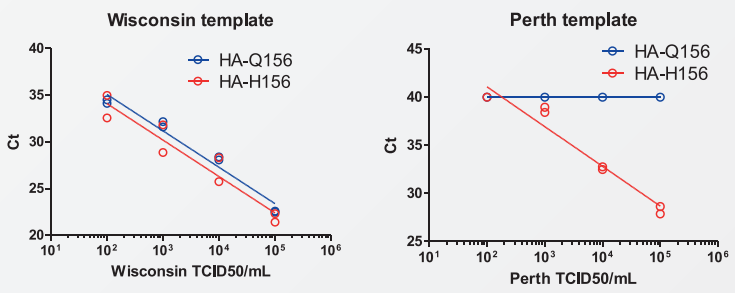
A/Perth/16/2009 influenza only has HA-H156 present and provides a useful control for the allelic specificity of the PCR reagents.



(M at 536 bp = adenine or cytosine)

We designed two probes to target the SNP at 536 bp: HA-Q156 recognises adenine and probe HA-H156 recognises cytosine. The probe is designed to position the mismatch within the intercalation region of the minor groove binding (MGB) moiety such that the combination of decreased probe length and lack of MGB binding affinity provides the molecular mechanism for 100% allelic discrimination (Kutyavin et al, 2000). The prediction would be for A/Wisconsin to be detected by BOTH probes but A/Perth/16/2009 to be detected ONLY by the HA-H156 probe.

Figure 2 . Total RNA was extracted from log dilutions of noted virus stocks, set up in triplicate using the allelic specific probes.



Our conclusion is that only the HA-H156 probe recognises A/Perth RNA and HA-Q156 does not bind at all as predicted. Note that the Ct values for both HA-H156 and HA-Q156 on A/Wisconsin extracted RNA are equivalent confirming the ~50:50 ratio of the two HA variants identified by sequencing.

HA-H156 and HA-Q156 exhibit altered viral profiles during infection.

Figure 3. Inoculum virus contains equivalent amounts of both variants.

A/Wisconsin/67/2005 was used to inoculate human volunteers in the hVCM as previously described (Bagga et al, 2013).

The inoculum virus was analysed in parallel to confirm that the HA variants were present in equivalent amounts **(Figure 3).**

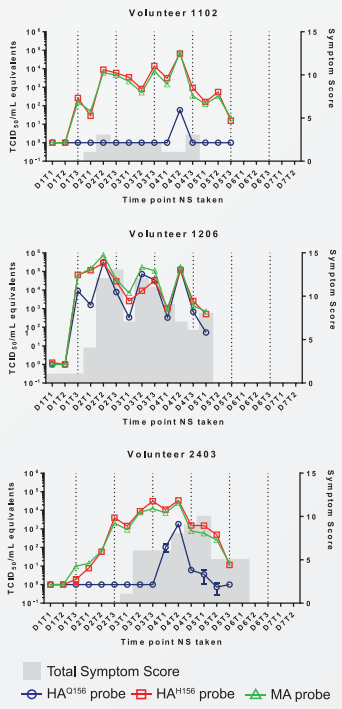


Figure 4. Viral shedding time course in volunteers successfully infected following A/Wisconsin/67/2004 virus challenge. Titres were determined by RT-qPCR using either HA-H156probes, HA-Q156 probes or matrix (MA) probes.

The viral shedding profile of 3 successfully inoculated human volunteers in shown in Figure 4. All 3 volunteers were inoculated at Day 0 and NPS samples were collected 3 times daily for volunteers 1102, 2403 and 1206. Each volunteer enters daily symptoms in a diary. The matrix (MA) probe is able to detect and quantitate both HAQ156 and HAH156 viral variants and therefore gives a measure of overall total viral load.

The HAH156 probe and the 'flu matrix (MA) probe gave essentially identical AUC data for TCID50/mL equivalents per day for both volunteers.

Strikingly, the egg adapted HAQ156 variant showed log10-fold reductions in viral RT-qPCR signal at early stages of viral challenge (day 1 mid-day and day 2 morning) for all volunteers. The effect is most evident in volunteers 1102 and 2403 with no replication of HAQ156 variant virus observed at day 2-3 despite peak viral loads observed for the HAH156 variant.

Summary

1. hVIVO has developed allele specific PCR assay technology with a high degree of specificity to track poly morphic HA variants during infection
2. A/Wisconsin/67/2005 grown on VERO cells has a 50:50 ratio of HA-H156 and HA-Q156 variants. The HA-Q156 variant is a well known egg adapted mutation.
3. The ratio of HA-Q156 egg adapted variant has a much lower viral load compared to the HA-H156 variant in the same inoculum bolus. Interestingly, the influenza symptoms map to the viral peak of the HA-Q156 variant and not the HA-H156.
4. The use of matrix RT-qPCR probes avoids the variation in measuring viral loads due to HA SNPs.
5. It is unclear whether the impact of the SNP at 156 is directly responsible for differential viral replication kinetics but we note the close proximity of this SNP to the HA receptor binding site (Koel et al, 2013).

References

1. Kutyavin I.V., et al (2000). 3'- Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res. 2000 January 15; 28(2): 655–661.

2. Bagga et al, 2013, Comparing influenza and RSV viral and disease dynamics in experimentally infected adults predicts clinical effectiveness of RSV antivirals. Antivir Ther. 18(6) 785-791

3. Koel et al, (2013) Substitutions near the receptor binding site determin major antigenic change during influenza evolution. Science 342 976-981.

Measurement of intracellular replicative strand in Respiratory Syncytial Virus (RSV) infected human volunteers.

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Background

The RSV life-cycle within infected airway epithelial cells utilises positive sense (+ve sense) RNA replicative intermediates (RIs) including antigenome and mRNA transcripts for replication and viral protein synthesis respectively. hVIVO have now developed a strand-specific RT-qPCR assay to quantify specifically the active intra-cellular +ve sense RSV RI present in successfully infected epithelial cells. We demonstrate specific detection of <0.1 % +ve sense RI RSV in 99.9% virion genomic RSV mixtures with a lower limit of quantitation of RI RSV at 10 copies/assay well. Furthermore we have used the strand specific RI RSV RT-qPCR assay to generate area-under-the-curve (AUC) profiles using the cellular component of nasal washes collected from infected human volunteers and significantly different profiles of RI RSV AUC profile compared to total RSV AUC. Furthermore, the RI RSV AUC closely resembles infectious viral AUC generated by cell-based plaque assay data.

Introduction

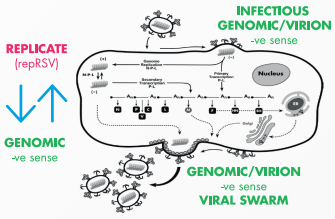


Figure 1: RSV replicative cycle

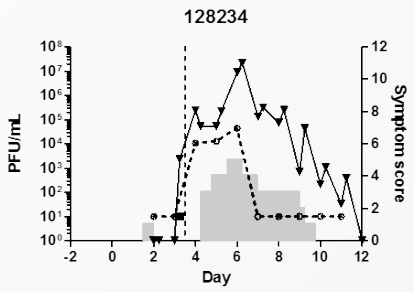


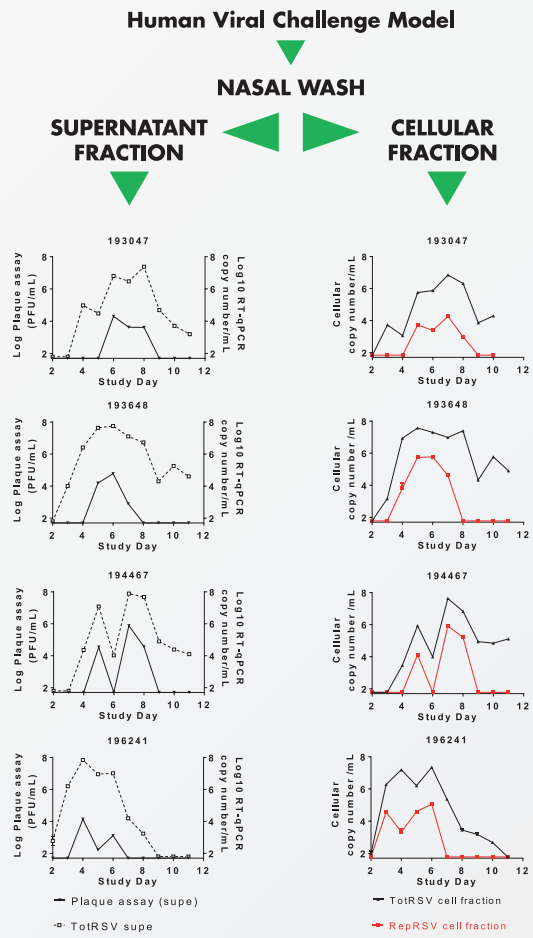
Figure 2: Nasal wash samples from RSV inoculated volunteers were analysed by RT-qPCR (solid line, filled triangles), and plaque assay (broken line, empty circles) and plotted against collection time. (shaded towers equals symptoms score.)

RSV replicates through +ve sense stand replicative RNA intermediates (RI) in infected patients (Figure 1).

The area-under-the curve (AUC) data for RSV replication in RSV infected human volunteers is measured by total RT-qPCR detecting mainly ex-tra-cellular genomic RNA (-ve strand). It is well-known that RSV AUC RT-qPCR curve has an exaggerated increase at late infection compared to plaque assay AUC data (Figure 2), This is caused by accumulation of non-inhibitible, replicative-defective particles. This late-stage AUC surge confounds antiviral efficacy determination of test compounds targeting RSV replication.

We hypothesise that measurement of intra-cellular RI RSV by RT-qPCR gives a fast, sensitive measure of anti-viral efficacy.

RI-RNA strand specific RT-qPCR assay in RSV infected humans



Nasal wash samples were collected from 4 RSV Memphis 37B inoculated healthy volunteers across study day 2 and day 12. Each plot is identified by the subject ID.

SUPERNATANT FRACTION
Total RT-qPCR (dotted line, □symbol) and plaque assay data (solid line,▼symbols).

CELLULAR FRACTION
Total RT-qPCR (solid line, ▲symbols) and RI specific RSV. (solid red line)

Both total RT-qPCR profiles (supernatant and cellular) show the characteristic high AUC late in infection (post day 8).

The RI-RSV AUC profile show a completely different profile with a lower maximum peak and rapid "cliff-face" fall-off late in infection. This profile is very similar to the plaque assay data.

Strand Specific reverse transcription (RT)-qPCR

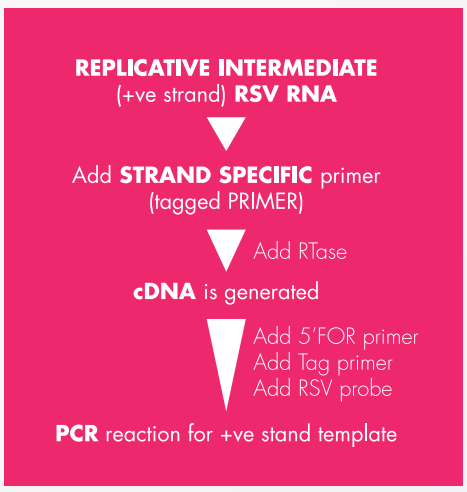


Figure 3 Scheme for strand specific RT-qPCR

Mixture	RI-RSV copies	Genomic RSV copies	Ratio RI/gen
1	10^1	10^4	1/1000
2	10^2	10^4	1/100
3	10^3	10^4	1/10
4	10^4	10^4	1/1
5	10^5	10^4	10/1
6	10^6	10^4	100/1
7	10^7	10^4	1000/1

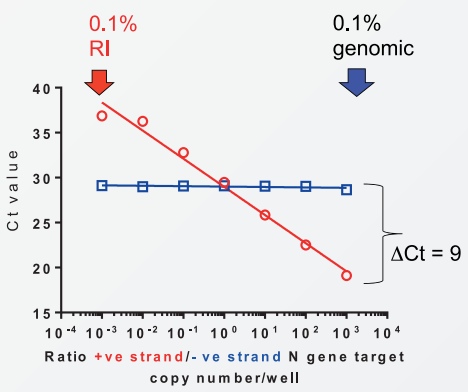


Figure 4: Strand specific RT-qPCR was performed on in vitro RNA mixtures at different ratios (see inset table).

In vitro RNA copies of the RSV target region were synthesised in both +ve sense (RI) and –ve sense (genomic) orientations. Mixtures were made to generate 0.1% RI in genomic RNA background to 0.1% genomic in RI background (see mixture columns). Strand-specific RT-qPCR was performed on all the mixtures (see Figure 4).
Note the Ct difference between the 0.1% RI and genomic is Ct = 9, as expected for 3 log concentration change in template demonstrating 100% SPECIFICITY STRAND DETECTION.
The lower limit of quantitation for the RI-RSV RT-qPCR assay is 10 copies/rxn

CONCLUSION

1. TWO independent anti-viral efficacy measurements from ONE clinical sample is possible.

Cell pellet → RI-RSV specific RT-qPCR
Supernatant → total RT-qPCR (totRSV).

2. RI-RSV AUC profile is very similar to plaque assay.

Replicative/mRNA RSV has short t½ and rapid turnover whereas the genomic RSV in extracellular virion is stable. The totRSV AUC in late stage infection likely due to accumulation of replication-incompetent or neutralised with bound IgG. Either way, the intra-cellular RI-RSV AUC appears to reflect behaviour of of replication competent virions plaque-forming RSV.

3. RI-RSV AUC metric for virus replication inhibitors.

Unlike the plaque assay; no confounding cytotoxic IMP effects will be observed in RT-qPCR format. The RI-RSV AUC may be a more sensitive metric for anti-viral efficacy. The RI-RSV assay has a faster turnaround time compared to plaque assay

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