Performance improvement of influenza A/H1N1pdm09 complete genome massive sequencing by setting a cycle threshold cut-off in monoplex PCR

I Sanz[1,2], F Pozo[4], A Antón[5], C Gulin[5], C Andrés[5], M Domínguez-Gil[6], I Casas[4], JC Galán[7], JM Eiros[1,2], S Rojo[1,2], MJ Rodríguez[7], T Pumarola[5], S Tamames[3], R Ortiz de Lejarazu[1,2]

1. Valladolid National Influenza Centre, Valladolid, Spain
2. Microbiology Service, University Clinic Hospital of Valladolid, Spain
3. Public Health Office. Government of Castile and Leon, Valladolid, Spain
4. Madrid National Influenza Centre, Instituto de Salud Carlos III, Madrid, Spain
5. Microbiology Service, University Hospital Vall d´Hebron, Barcelona, Spain
6. Microbiology Service, University Hospital Rio Hortega, Valladolid, Spain
7. Microbiology Service, Hospital Ramón y Cajal, Madrid, Spain

Introduction & Objective

The increasing use of Next generation sequencing (NGS) for diagnosis and research needs a continue optimization for reducing the cost of each test and for obtaining the greater scientific and economic benefits. The aim of this study is to determine the minimum relative viral load that should have a positive respiratory samples for A/H1N1pdm09 influenza virus for obtaining the best performance in NGS

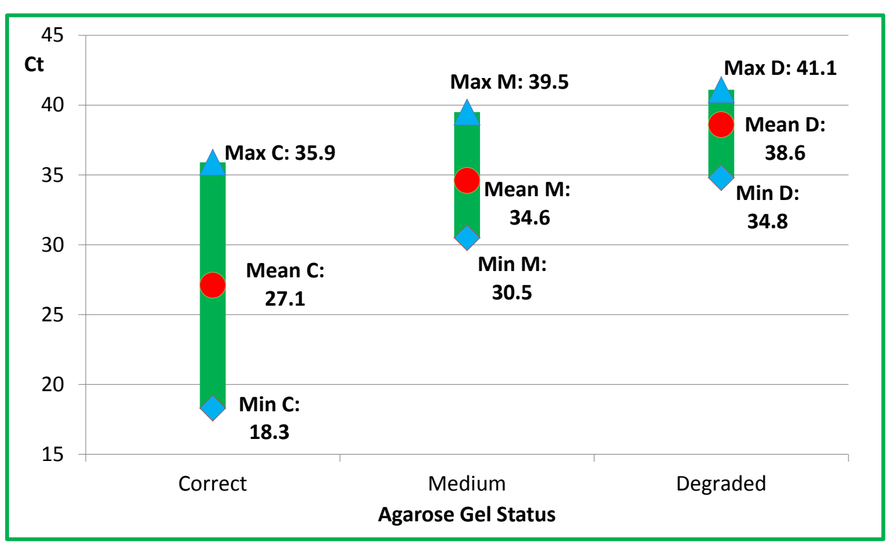

Figure 1. Maximun, minimum and mean Cts obtained from samples that showed the different gel status described. (C-Correct gel; M-Medium gel; D-Degraded gel)

Material/methods

- A Prospective multicentric study was performed recruiting 75 upper and lower respiratory samples from patients diagnosed for A/H1N1pdm09 flu virus during 2013-14 and 2014-15 influenza epidemics.
- Viral load was measured as Cycle Threshold (Ct). Analysis was performed by a real time monoplex RT-PCR using *ABI 7500-Fast* platform (*Applied BioSystems*) and using the A/H1N1pdm09 HA primers and probes from the influenza CDC subtyping reagents (IRR reference FR-929).
- Before sequencing, the 8 flu amplicons were generated using PathAmp™ FluA reagents. Quality of PCR products was tested performing 2% agarose gels to check the integrity of gene fragments before sequencing, defining the following categories: Correct (C) (visualization of the 8 fragments); Medium (M) (less than 8 fragments); Degrade (D) (without apparent bands).
- Massive Sequencing was done using *Ion Torrent PGM* (*Life Technologies*). Acceptation intervals were designed using the maximun, minimum and the average Cts values from all samples.

Results

Average Ct of samples that showed Correct gels was 27.1 (Max:35.9; Min:18.3), 34.6 for Medium gels (Max:39.5; Min:30.5) and 38.6 for Degraded (Max:41.1; Min:34.8) (Figure 1). It was observed significant differences between the Ct mean of these three groups (Student-T; $p<0.01$). It was observed nucleotide gaps and poliNs in some sequences from samples that obtained Medium gels with Cts higher than 36.0, and in some of theme the sequencing of PB1, PB2 and PA genes was impossible. All samples with Correct and Medium gels with Cts lower than 36.0 were correctly sequenced. With these results, Ct 36.0 was established as the cut-off for sequencing procedure. This cut-off was later tested in 21 new samples obtaining Correct gels in all of theme, with high quality of all sequences obtained.

Conclusions

Implementation of a minimum relative viral load of A/H1N1pdm09 influenza virus can increase the performance of massive sequencing techniques, reducing the cost of thereof. To obtain the maximum efficiency of sequencing influenza with Ion Torrent it is necessary a Ct of at least 36.0 for obtaining complete and high quality sequences. For this reason it is not recommended to include clinical samples with relative viral loads lower than this value in this assays.