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RegaVir platform: Case discussions antiviral resistance testing

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A European multi-centre External Quality Assessment (EQA) study on phenotypic and genotypic methods used for Herpes Simplex Virus (HSV) drug resistance testing

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A European multi-centre External Quality Assessment (EQA) study on phenotypic and genotypic methods used for Herpes Simplex Virus (HSV) drug resistance testing

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Why a European multi-center EQA study?

- There are a **small number of specialized laboratories across Europe** that offer HSV drug resistance testing with limited quality assurance resources.

- At the time of the study was conducted,
  - the first pilot scheme for HSV genotyping has just concluded (distributed by www.qcmd.org), **QCMD** (**Q**uality **C**ontrol for **M**olecular **D**iagnostics)
  - no proficiency panel for phenotyping available

- **First combined European EQA scheme for HSV resistance testing, reporting on both genotypic and phenotypic assays.**

Objectives

- To coordinate the first European HSV EQA scheme, enabling:
  i. the evaluation of phenotypic and genotypic methods used for HSV drug resistance testing in specialized laboratories
  ii. the comparison of genotypic, phenotypic and clinical reports between participating laboratories
  iii. the establishment of a network of collaborating laboratories for ongoing quality assurance

Study design

- 5 participant laboratories

Country	Center
Belgium	Rega Institute for Medical Research, KU Leuven, Leuven, Belgium; Laboratory of Virology, RegaVir
France	Hospices Civils de Lyon, Lyon
France	National Reference Centre for Herpesviruses, La Pitié Salpêtrière-Charles Foix University Hospital, Paris
Ireland	Microbiology Department, Central Pathology Laboratory, St James's Hospital, Dublin
UK	Virology Reference Department, Public Health England (PHE), Colindale, London

Study design

- The testing panel was prepared from UK clinical samples submitted between 2010 and 2013 to the Antiviral Unit (AVU), Virus Reference Department (VRD), Public Health England (PHE), Colindale

Sample	HSV type	Age/Sex	Clinical notes	Treatment history	Sample	Viral load (copies/ml)	Virus titer (PFU/ml)
HSV-EQA-2016-A	1	33 / F	Eczema herpeticum	ACV	Swab	6.1×10^4	3.1×10^3
HSV-EQA-2016-B	2	36 /M	Immunosuppressed	Unknown	Ulcer swab	5.3×10^4	2.2×10^2
HSV-EQA-2016-C	1	7 / M	Ulcer on tongue	Unknown	Ulcer swab	6.9×10^4	2.1×10^3
HSV-EQA-2016-D	2	19 / F	Post-allograft BMT	ACV, FOS	Genital swab	4.9×10^4	1.7×10^2
HSV-EQA-2016-E	2	36 / M	Immunosuppressed	Unknown	Ulcer swab	6.7×10^3	2.2×10^1

Sample HSV-EQA-2016-E comprised a 10x dilution of sample HSV-EQA-2016-B in order to help assess assay sensitivity. The five samples (referred hereafter as EQA-A through EQA-E) were quantified by qPCR (Geneproof), diluted in Dulbecco's Modified Eagle Medium and stored at −80 °C as 1 ml aliquots in sealed bags prior to distribution.

Study design

- Participants simultaneously received the panel on dry ice.
- Six-week deadline for return of results.
- Labs were free to process the samples according to their local protocols.

  (Unlike most EQA schemes, where large parts of how participants analyze and report their data return are strictly specified, no such prescriptions were made for this evaluation).
- Centers had to report:
  - Phenotypic and genotypic data
  - Responses to a questionnaire describing methodologies, viral strains and reference viral sequences.

Results

- Four out of five laboratories (coded 1 through 4 to preserve anonymity) returned data sets within the allotted turnaround time.

- The fifth center returned partial data sets later, which were not included in the analysis.

Results: genotypic assays

- From the questionnaires, each assay methodology was unique, but all conformed to the basic principle of measuring viral growth in cell monolayers at different dilutions of drug and establishing the drug concentration at which a specific level of viral growth inhibition occurs, commonly 50% or 95% (EC_{50} or EC_{95}).

- **Each lab used its own reference strains, cell lines and cut-off values for drug susceptibility**, but all used the standard multi-well plate format.

Details of each centre's phenotypic assay. Resistance cut-offs are measured in µM unless followed by a 'x', in which cases they are fold-change over wild-type.

Center	Phenotypic assay	Cell line	Reference strains	Resistance cut-off EC_{50}'s (HSV-1)					Resistance cut-off EC_{50}'s (HSV-2)			
				ACV	PCV	BVDU	FOS	CDV	ACV	PCV	FOS	CDV
1	PRA	Vero	Sc16, 333	3.0	10	NT	250	24	6.5	38	250	NT
2	Dye-uptake	Vero	KOS, ROIZ, DM21	6.5	NT	NT	350	NT	13.5	NT	350	NT
3	CPE	HEL	KOS, G HSV2	10x	10x	10x	3x	3x	10x	10x	3x	3x
4	PRA	Vero	KOS, G HSV2	7.0	NT	NT	330	NT	7.0	NT	330	NT
5	No information shared											

PRA: Plaque Reduction Assay, whereby cytopathic effect (CPE) is quantified by microscopically counting the number of viral plaques formed

CPE (Cytopathic Effect) reduction assay, whereby each well is assigned a level of CPE from 0 to 5 (0 corresponding to 0% CPE and 5 to 100% CPE), also after microscopic examination of wells

NT: Not tested.

Results: phenotypic resistance

- Although EC_{50} data was available from testing labs, the variations in assay design precluded direct comparison

  ⇒ the clinically reported susceptibility levels of either 'Sensitive', 'Intermediate' or 'Resistant' to each drug were compared.

- Direct comparison of each lab's results showed concordance for most sample-drug combinations (variation between intermediate and resistant reporting was not considered noteworthy).

Results: phenotypic assays

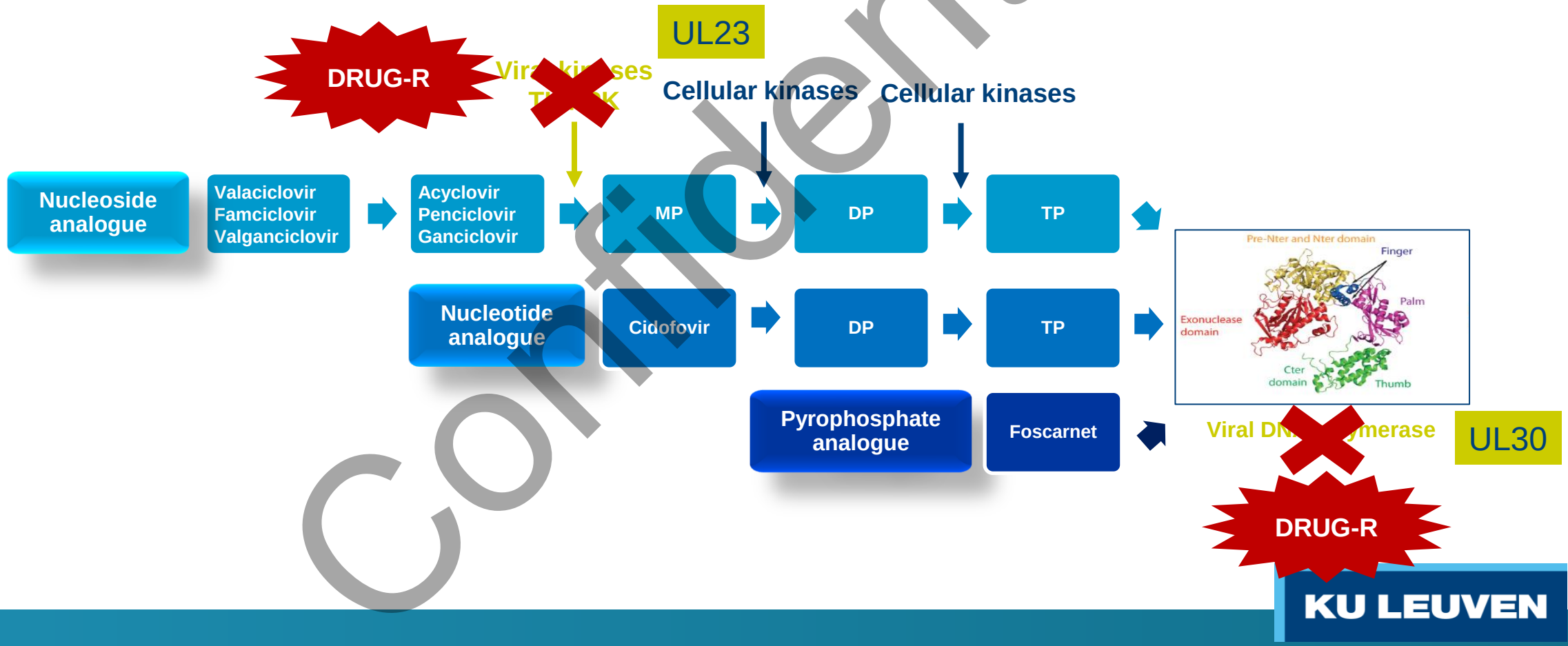
Sample	Drug	Lab 1	Lab 2	Lab 3	Lab 4
EQA-A	ACV	S	S	S	S
	PCV	S	–	S	–
	CDV	S	–	S	–
	FOS	S	S	S	S
EQA-B	ACV	R	R	R	R
	PCV	R	–	R	–
	CDV	–	–	S	–
	FOS	S	S	S	S
EQA-C	ACV	I	R	I	R
	PCV	I	–	S	–
	CDV	S	–	I	–
	FOS	R	R	R	R
	FOS	I	R	R	R

Results: phenotypic assays

Sample	Drug	Lab 1	Lab 2	Lab 3	Lab 4
EQA-D [a]	ACV	I	S	I/R	R
	PCV	R	–	I/R	–
	CDV	–	–	S/I	–
	FOS	I	R	R	R
EQA-E	ACV	R	R	R	R
	PCV	R	–	R	–
	CDV	–	–	S	–
	FOS	S	S	S	S

HSV genotypic resistance

- Genotypic resistance assays amplify and sequence UL23 and UL30, the two viral genes in which nearly all mutations conferring resistance to currently available drugs are located.

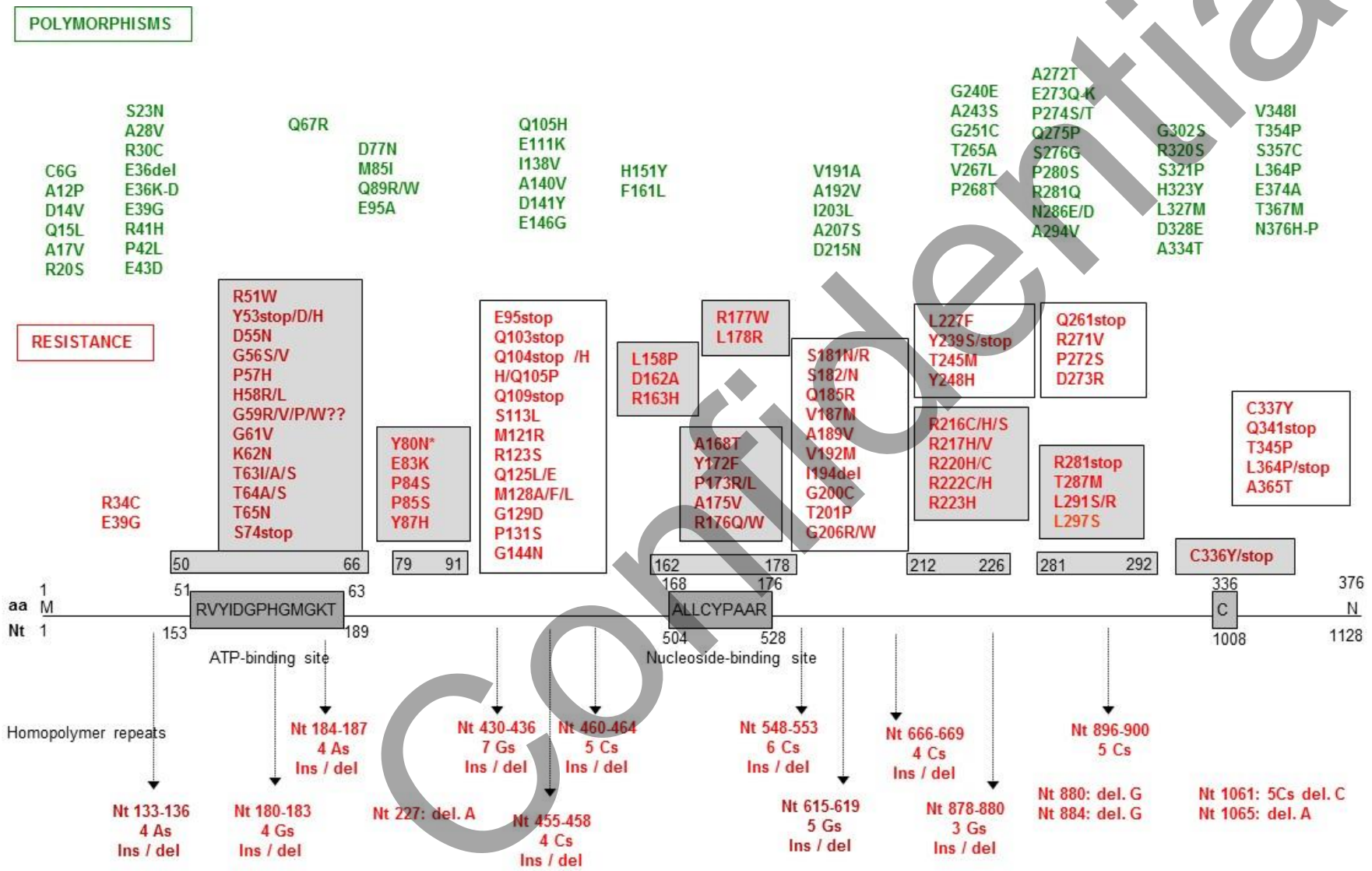


HSV genotypic resistance

- After amplification by PCR, complete viral genes (three labs) or regions encompassing the relevant domains (one lab) are sequenced using either Next Generation Sequencing, NGS (one lab) or more commonly, Sanger sequencing platforms (three labs).

- Loci where the consensus nucleotide sequence varies from a reference strain are classified as resistance-associated, polymorphic, or unclear, after consultation with collated list(s) of known mutations and their effects on antiviral susceptibility.

HSV-1 thymidine kinase (UL23)



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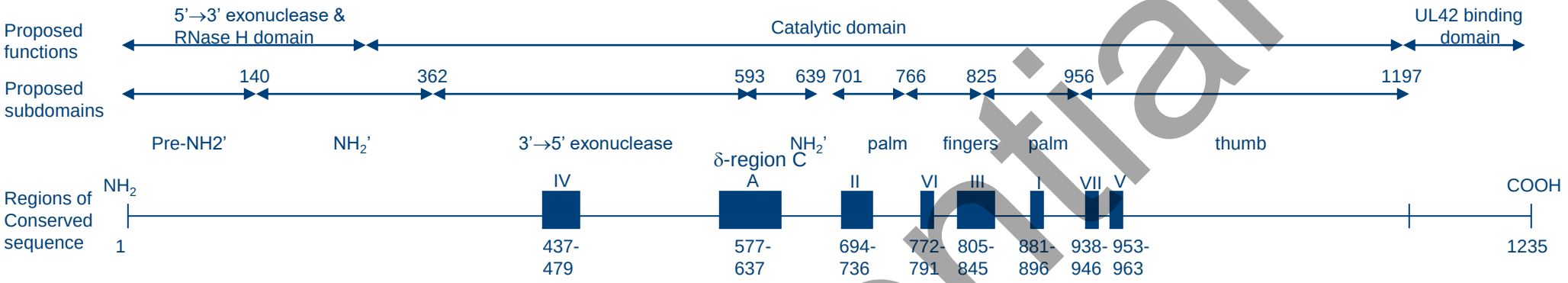
HSV-1 DNA polymerase (UL30)



Mutations known to confer altered sensitivity to ACV, PFA and/or aphidilcolin. (*hypersensitivity to ACV)

D368A E370A V462A K532T Y557S Q570R L583V Y577H* D581A* E597K/D A605V Y696H R700G V715G/M A719V/T S724N L774F L778M D780N L782I P797T V813M N815S/T N815L/V/Y/E Y818C T821M G841S/C R842S Y941H N961K S889A F891C/Y V892S

Mutations lethal to the virus

E460D G464V K522E P561S

Novel mutations selected *in vitro* under PFA or PME derivatives and characterized in our Laboratory

Q618H V714M L802F T821M R959H D1070N

Mutations selected *in vitro* under HPMP derivatives and characterized in our Laboratory

V573M R700M K960R W998L L1007M I1028T

Mutations found in clinical isolates associated with drug-resistance found in our previous studies.

H386Y A542S E798K I922T

Genetic polymorphisms

G12E A20V F23I A27T S33G E70D D72N A78V/D A102T K104Q D114A R116H G132C P137Q A138E F171S A229T A232T F248L R330A S338L I352V E421D N425T T434M N522S A566T T639I A646T D660E E684D D672N E675A D703N R700K V743M N711K G749D L753M P875S A899V V904M V905M M906V K908R P920S A995F E1005K I1026S E1082K A1099T D1103H E1104D S1123L-C P1124H A1128V G1129V L1166W E1194D P1199Q A1203T A1204T T1208A A1209T T1219M

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Results: genotypic resistance

- Reference strains and assay details for each center's genotypic assay

Center	UL23 & UL30 Sequencing	Sequencing method	Reference strains	Reference strain HSV-1		Reference strain HSV-2	
				Name	Genbank	Name	Genbank
1	Complete	NGS	Sc16, 333	Strain 17	JN555585	333	KP192856
2	Partial DNA pol: amino acids 409–979 of HSV-1 - HSV-2 pol was not sequenced	Sanger	KOS, ROIZ, DM21	Sc16	X03764	333	V00466
3	Complete	Sanger	KOS, G HSV2	KOS	JQ673480.1	gHSV2	KU310668.1
4	Complete	Sanger	KOS, G HSV2	Strain 17	X14112	HG52	Z86099
5	No information shared						

Detection and relevance of non-polymorphic sites should be independent of the reference sequence used by a laboratory.

Results: genotypic resistance

Sample	Gene	Lab 1	Lab 2	Lab 3	Lab 4
EQA-A	TK	None	None	None	None
	pol	None	None	None	None
EQA-B	TK	Del C (nt 551–556)	Del C (nt 551)	Mixed population at nt 551–556 Del C & wild-type	Del C (nt 551)
	pol	None	–	None	None
EQA-C	TK	None	None	None	None
	pol	S724N	S724N	S724N	S724N
EQA-D	TK	None	None	T288M*	None
	pol	R628G	–	R628G	R628G

Sample	Drug	Lab 1	Lab 2	Lab 3	Lab 4
EQA-D [a]	ACV	I	S	I/R	R
	PCV	R	–	I/R	–
	CDV	–	–	S/I	–
	FOS	I	R	R	R

Drug-susceptibility profile of a TK T288M mutant

Strain	EC_{50} (µg/ml)				
	Acyclovir	Ganciclovir	Brivudin	Foscavir	Cidofovir
RV-522 (DNA pol wt & TK T288M)	**>50** **22.4** **15** **29.2**	**5.0** **4.0** **2.0** **4.5**	>10 >10 >10 >10	10.1 25.3 16 16	0.22 0.19 0.2 0.2
	Mean FC = ≥662.5	**Mean FC = 1409**		Mean FC = 0.7	Mean FC = 0.2
Reference HSV-2 G strain	0.044	0.011	>10	25.3	0.60
EC_{50} : Concentration required to reduce virus induced cytopathicity by 50%					

Phenotyping and genotyping of 11 plaque-purified viruses isolated from EQA-D by lab 3.

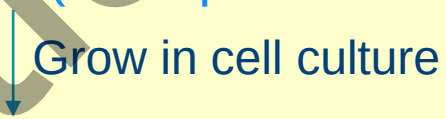
Clone	TK	pol	EC_{50} (µg/ml) & (fold change over the reference)			
			ACV	PCV	FOS	CDV
1	T288M	R628G	**20 (>417)**	**>20 (>83)**	**25.26 (3.2)**	0.88 (1.2)
2	T288M	R628G	**>20 (>417)**	**>20 (>83)**	**30.59 (3.8)**	1.16 (1.6)
			>20 (>213)[a]	**>20 (>83)**[a]	**30.59 (3.8)**[a]	***0.84 (2.6)***[a]
3		R628G	*0.42 (8.8)*	*0.58 (2.4)*	**81.79 (10.2)**	1.16 (1.6)
4		R628G	*0.47 (9.8)*	*0.53 (2.2)*	**63.35 (7.9)**	**2.74 (3.8)**
5		R628G	*0.47 (9.8)*	*0.80 (3.3)*	**81.79 (10.2)**	**3.58 (5)**
6		R628G	*0.36 (7.5)*	*0.53 (2.2)*	**40.00 (5.0)**	**2.09 (2.9)**
7		R628G	*0.30 (6.3)*	*0.80 (3.3)*	**63.35 (7.9)**	1.22 (1.7)
8[a]		R628G	*0.40 (4.3)*	*0.36 (1.5)*	**40.00 (5.0)**	**1.07 (3.3)**
9[a]		R628G	*0.44 (4.7)*	*0.44 (1.8)*	**73.14 (9.1)**	**0.88 (2.8)**
10[a]	T288M	R628G	**>20 (>213)**	**>20 (>83)**	**23.39 (2.9)**	**1.13 (3.5)**
11[a]		R628G	*0.3 (3.2)*	*0.61 (2.5)*	**89.44 (11.2)**	**1.16 (3.6)**
HSV-2 ref (G)	–	–	0.048	0.24	8.0	0.72
			0.094[a]	0.24[a]	8.0[a]	0.32[a]

Bolded values are resistant; italicized values are intermediate.

[a] Clones 8–11 and the second set of results for clone 2 and the reference were performed on a different date to the others.

EQA-D analysis

- EQA-D (original sample): (TK T288M + DNA pol R628G) + (DNA pol R628G)



1 (DNA pol R628G)

- Following growth of the original sample in cell culture, the double mutant virus was overgrown by the DNA pol R628G mutant virus.

- Indeed, genotyping of the viral stock (#1 in cell culture) showed only the DNA pol R628G substitution.

⇒ These results suggest that the double-mutant virus has a reduced fitness.

Conclusions

- The gold standard method for detecting HSV resistance is a phenotypic assay, which requires specialized laboratory experience and is technically demanding with long turnaround times of up to three to four weeks.

- In contrast, genotypic tests can be performed within a few days and can be easily set up by most clinical microbiology laboratories with molecular experience but rely on data generated by phenotyping.

- ISO15189 standard 5.6.3 requires that laboratories participate in an inter-laboratory comparison or proficiency testing program.

- First European HSV EQA study to evaluate phenotypic and genotypic methods used for HSV drug resistance testing in five specialized laboratories from four European countries.

Conclusions

- Complete concordance was observed for sample EQA-A, which was sensitive to all drugs, and for the duplicate samples EQA-B and EQA-E, which contained a well-characterized deletion mutation in the HSV-2 TK gene resulting in a premature stop codon.

- Discordant interpretations were drawn for the other samples, both of which contained substitution mutations in the DNA pol and/or TK genes, which can confer variable effects on antiviral resistance.

- Both in vitro studies and clinical case reports associate the pol mutation S724N with resistance to ACV and FOS, an association supported by all four reporting labs in this study. However, discordant reports of susceptibility and low-level resistance to CDV and PCV by labs 1 and 3.

Conclusions

- Despite being the DNA pol R628G an unknown mutation, labs 1, 3 and 4 reported this change as being resistance associated.

- All three laboratories reported similar circumstantial evidence to justify this declaration:
  - R628G was the only unknown mutation in TK or pol that might account for ACV & FOS resistance
  - lies within the conserved delta-C region
  - R628C was previously reported as associated with ACV-R

Lab 3 had previously encountered the R628G mutation in a resistant isolate.

Conclusions

- **EQA-D:** inability of all but one participant to detect the resistance-associated TK mutation - value of its inclusion in this exercise.

- Lab 1 detected a mixed base at position 863 using NGS, but failed to report the expected translation of T288 M. Instead, apparently **due to mis-numbering of the alignment with the reference strain**, this was reported at position 287, leading to its omission from reported resistance loci.

- Lab 1 failed to report genotypic resistance despite successfully sequencing the mixed population → interpretation difficulties due to artefacts generated by the **NGS methodology and accompanying bioinformatics pipeline**, highlighting the **need for rigorous validation of these techniques in diagnostic settings.**

- Accurate detection of the multidrug-resistant profile of EQA-D would be of critical importance in the patient's clinical management.

Other EQAs

Organizer (year)	Original ID	RegaVir ID	HSV type	Genotyping	Phenotyping
Hôpital de la Croix Rousse, Lyon, France (2016)	EIL-HSVR-2016-A	RV-1000	HSV-2	TK: wild-type DNA pol: wild-type	Wild-type
	EIL-HSVR-2016-B	RV-1001	HSV-2	**TK: L158P** DNA pol: wild-type	**ACV-R, PCV-R, GCV-R**
	EIL-HSVR-2016-C	RV-1002	HSV-1	TK: wild-type (**A365V**) **DNA pol: S724N** (**S127L,F205C**)	**ACV-R, PFA-R,** CDV-I
Hôpitaux Universitaires La Pitié Salpêtrière, Paris, France (2017)	EIL-HSVR-PSL2017-1	RV-1201	HSV-1	**TK: C del. Nts 1061-1065_mixed** DNA pol: wild-type	**ACV-R, PCV-R, GCV-R**
	EIL-HSVR-PSL2017-2	RV-1202	HSV-2	TK: wild-type DNA pol: wild-type	Wild-type
	EIL-HSVR-PSL2017-3	RV-1203	HSV-2	**TK: G del. Nts 837-840** **DNA pol: F738S**	**ACV-R, PCV-R, GCV-R, PFA-R, CDV-R, ADV-R**
Hôpitaux Universitaires La Pitié Salpêtrière, Paris, France (2018)	EIL-HSVR-PSL2018-1	RV-1441	HSV-1	TK: wild-type DNA pol: wild-type	Wild-type
	EIL-HSVR-PSL2018-2	RV-1442	HSV-1	**TK: Q250 stop** DNA pol: wild-type (**H408Y**)	**ACV-R, PCV-R, GCV-R, BVDU-R**
	EIL-HSVR-PSL2018-3	RV-1443	HSV-2	**TK: R223C** DNA pol: wild-type (**R604H**)	**ACV-R, PCV-R, GCV-R**

Underlined: novel mutations