AUTOMATED, TILT-FREE INTERPRETATION OF HEMAGGLUTINATION INHIBITION (HAI) ASSAYS WITH CYPHER ONE

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Abstract

Hemagglutination (HA) and hemagglutination inhibition (HAI) assays have been utilized for 70+ years and play a critical role in influenza vaccine development. In particular, HAI is critical in antigenic characterization of flu viruses and in evaluating immunogenicity of cell-based and traditional egg-based vaccines. HAI assays are prone to poor lab-to-lab consistency due to subjectivity in interpretation between human "readers" where a difference in endpoint of ±1 dilution (typically, ~4x change in concentration) is often considered "equivalent". In addition, the presence of non-specific inhibition (NSI) can further complicate analysis. To aid in the interpretation of samples which exhibit NSI, human readers will commonly "tilt" the plate at a 45^{o} angle for 30-60 seconds and look for a "tear drop" formation of the settled red blood cells (RBCs). Here we investigate whether the use of an automated imaging and interpretation system, the Cypher One, can generate accurate results without the need to "tilt" the plate.

We compared performance of the Cypher One automated interpretation to the visual interpretation of a human expert reader for a HAI dataset of 2200 samples. The samples consisted of de-identified human sera subjected to H1, H3 and B influenza strains. This particular experiment utilized both avian and mammalian RBCs for determining the serological response of samples in H1/B antigens or H3 antigens, respectively. Although the mammalian RBC subset did not require plate tilting prior to the human interpretation, all plates within the avian RBC subset did require tilting by the human reader. The Cypher One automated titer interpretation was obtained without tilting and compared to the interpretation obtained by the human reader.

The comparison yielded 95.6% agreement between the expert reader and automated interpretation method (within ± 1 dilution) for the complete dataset. Importantly, ~25% of clinical samples within the avian RBC subset exhibited NSI, which is known to complicate the manual interpretation and is often cited as a cause for the need to tilt the plate for accurate interpretation. Even in the presence of NSI, Cypher One showed high agreement (94.3% ± 1 dilution) without the need to tilt the plate for the challenging avian subset. The Cypher One system thus achieved high accuracy and consistency while providing the additional benefit of a digital database of plate images and associated results to meet data integrity requirements.

HAI Assay: Background

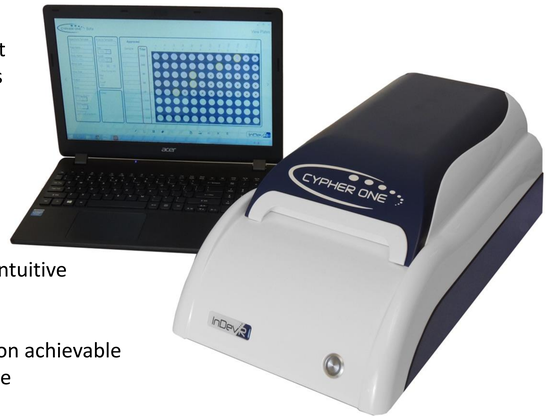
- **Hemagglutination:** HA proteins of influenza viruses cross-link red blood cells (RBCs) of avian or mammalian origin through binding to sialic acids on the cell surface, resulting in sustained suspension of RBCs in solution.
- **Hemagglutination Inhibition (HAI) Assay:** titrated antibodies are added along with a fixed antigen concentration to determine the endpoint where hemagglutination is inhibited by the presence of the antibodies. This inhibition is visually identified as the red blood cells (RBCs) in solution precipitate, resulting in a solid red "button" at the bottom of the well.
- **HAI Interpretation:** The type of visual examination by a human reader may change based on RBC donor or plate type. For example, when using avian RBCs, it is common practice to tilt the plate at a 45^{o} angle to assign a titer value; whereas when using mammalian RBCs, the plate is not required to be tilted.
- **Problem:** Lack of standardized reagents and consistent technique combined with unpredictable presence of non-specific inhibitors that challenge the interpretation translate to high inter-lab and inter-reader variation.

Fully Agglutinated

Agglutination Inhibited (Non-Agglutinated)

Cypher One: Automated Solution

- High fidelity imaging of HA and HAI assays
- Standardizes assay interpretation with an innovative analysis algorithm
- Rapid analysis provides consistent and reliable results
- Enhanced data integrity with a traceable digital record
- User-friendly and intuitive software interface
- ~2x image resolution achievable with the human eye



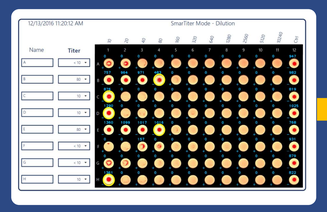
- Features to enable compatibility with 21 CFR Part 11/Annex 11
- Adjustable parameters can be optimized for various sample/plate types

Method

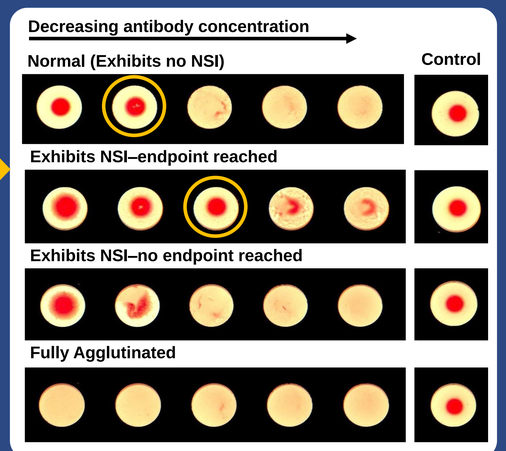
- 2,200 blinded human serum samples analyzed by HAI against 12 different antigens (H1/B and H3 strains)
- All samples underwent a standard receptor destroying enzyme (RDE) treatment
- Serum serially diluted (2-fold) across the row (i.e. A1-A11) in U-shaped 96-well plates
- 0.5% Turkey RBCs were added to all wells investigating the response of H1 and B influenza strains; 0.75% Guinea Pig RBCs were added to all wells investigating H3 influenza strains.
- Column 12 used as a non-agglutinated negative control (no serum, no virus)
- Cypher Imaging: Each plate was first imaged in the flat orientation (not tilted) using Cypher One and immediately transferred to the experienced human reader for analysis
- Human Interpretation: The experienced human reader placed each plate at a 45^{o} angle for approximately 1 minute prior to making an interpretation
  - The final titer value (inverse of the dilution factor) was assigned as the last non-agglutinated dilution within the series.
  - In cases for which all wells within the row were fully agglutinated (+) or when non-specific inhibition was detected, a HAI titer of 5 was arbitrarily assigned because the initial serum dilution was 1:10

Results and Discussion

- The Cypher One image analysis algorithm evaluates the extent of agglutination to determine and display the titer.
- The initial analysis yielded a high degree of samples exhibiting non-specific inhibition (NSI) type characteristics.



- A careful examination of all images collected with the Cypher One was performed to classify each sample into four distinct categories based on the morphological response.



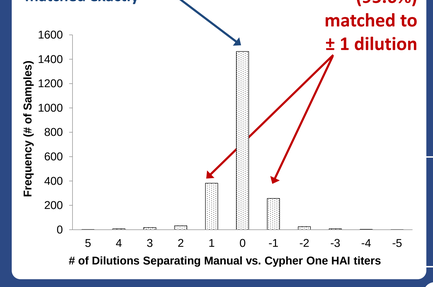
RBC Type	Classification	N	% Agreement (+/- 1 Dilution)
Turkey	Normal (No NSI)	591	97.5%
	NSI - endpoint reached	236	90.3%
	NSI - no endpoint reached	299	89.3%
	Fully Agglutinated	112	100.0%
Guinea Pig	Normal (No NSI)	790	98.2%
	NSI - endpoint reached	33	87.9%
	NSI - no endpoint reached	91	90.1%
	Fully Agglutinated	48	100.0%

- Classifications where any NSI is present (***NSI – endpoint reached*** and ***NSI – no endpoint reached***) are challenging to interpret even for experienced readers.
- The turkey RBC dataset had a large percentage (**43.2%**) of samples that were categorized as "challenging"; whereas the guinea pig dataset had only **12.9%**.

An agreement within ± 1 dilution or a 2-fold difference from the reference titer is considered an equivalent result due to the variability in the assay and interpretation expected between users.

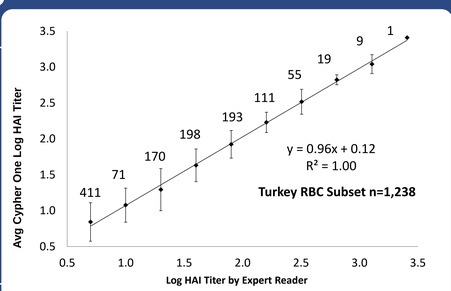
- Both **B/Florida/78/2015** strains (cell and egg) showed the lowest overall agreement, and also had the largest combined number of samples that were classified as exhibiting NSI (either endpoint reached or no endpoint reached).

RBC Type	Antigen	N	% Agreement (+/- 1 Dilution)
Turkey	1 (H1N1 A/Michigan/45/2015)	193	99.5%
	2 (B/Florida/78/2015) - Cell	94	90.4%
	3 (B/Brisbane/60/2008)	193	94.3%
	4 (B/Florida/78/2015) - Egg	193	90.7%
	5 (B/Arizona/10/2015)	190	92.6%
	6 (H1N1 A/California/07/2009)	193	94.8%
	7 (B/Phuket/3073/2013)	182	96.2%
	Combined Subset Total	**1238**	**94.3%**
Guinea Pig	8 (H3N2 A/Alaska/232/2015)	190	98.4%
	9 (H3N2 A/Texas/88/2016)	193	95.9%
	10 (H3N2 A/Hong Kong/4801/2014)	193	95.9%
	11 (H3N2 A/Switzerland/9715293/2013)	193	94.8%
	12 (H3N2 A/Hong Kong/4801/2014)	193	96.4%
	Combined Subset Total	**962**	**97.2%**



- Overall, 95.6% the titer calls made by Cypher One were accurate to within +/- 1 dilution of the manual titer call, indicating a high level of agreement to the experienced human reader.

- A linear regression of the Turkey RBC data has a slope of 0.96 and a Pearson's correlation coefficient of 1, indicating a strong linear correlation between the two interpretation methods.
- The number above each data point in the figure is the total number of samples with a specific titer call as defined by the experienced reader titer value.



Acknowledgements/Publication:

- We gratefully acknowledge funding from NIH/NIAID (R44 AI106054).
- We also gratefully acknowledge Drs. Zhiping Ye and Hang Xie at FDA for their contributions.
- Wilson, Garrett et al. "Automated interpretation of influenza hemagglutination inhibition (HAI) assays: Is plate tilting necessary?" PloS one vol. 12,6 e0179939. 29 Jun. 2017, doi:10.1371/journal.pone.0179939