China-Kazakhstan "One Belt One Road" Equine Infectious Disease Prevention and Control

Laboratory Diagnosis Technology of Equine Influenza

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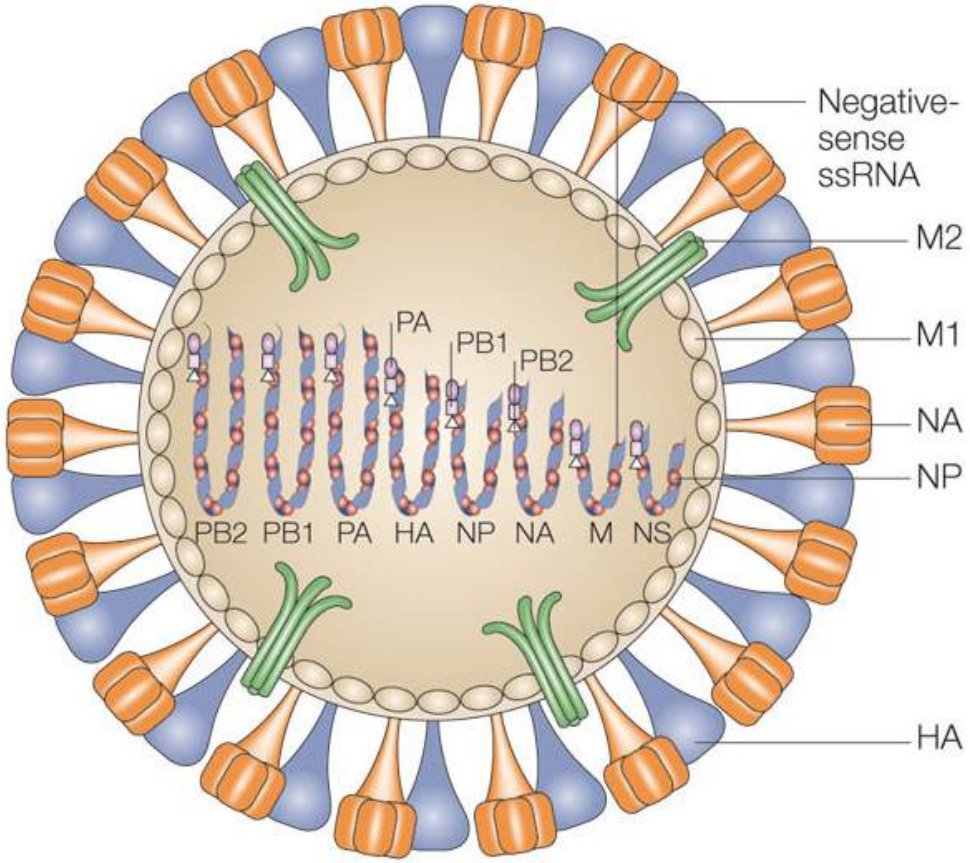
Equine Influenza

Equine influenza, is an acute outbreak of infectious diease among equine animals caused by the EIV (Equine influenza Virus) of the influenzavirus of the Orthomyxoviddae.

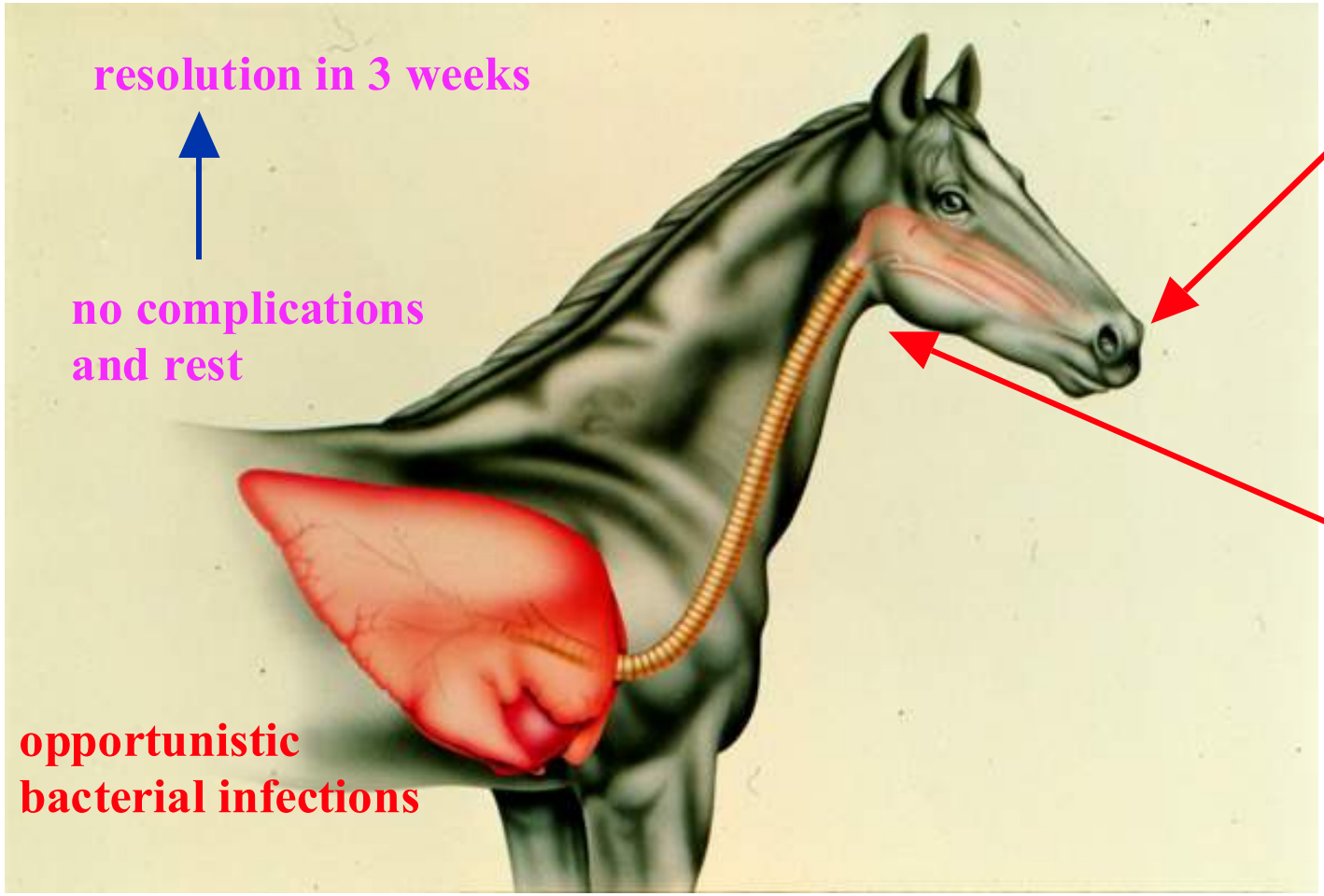
- Envelope protein：HA、NA
- Matrix protein：M1、M2
- RNA polymerase：PB2、PB1、PA
- Nucleoprotein：NP
- Non-structural protein：NS1、NS2



(Equine)：
H7N7
H3N8

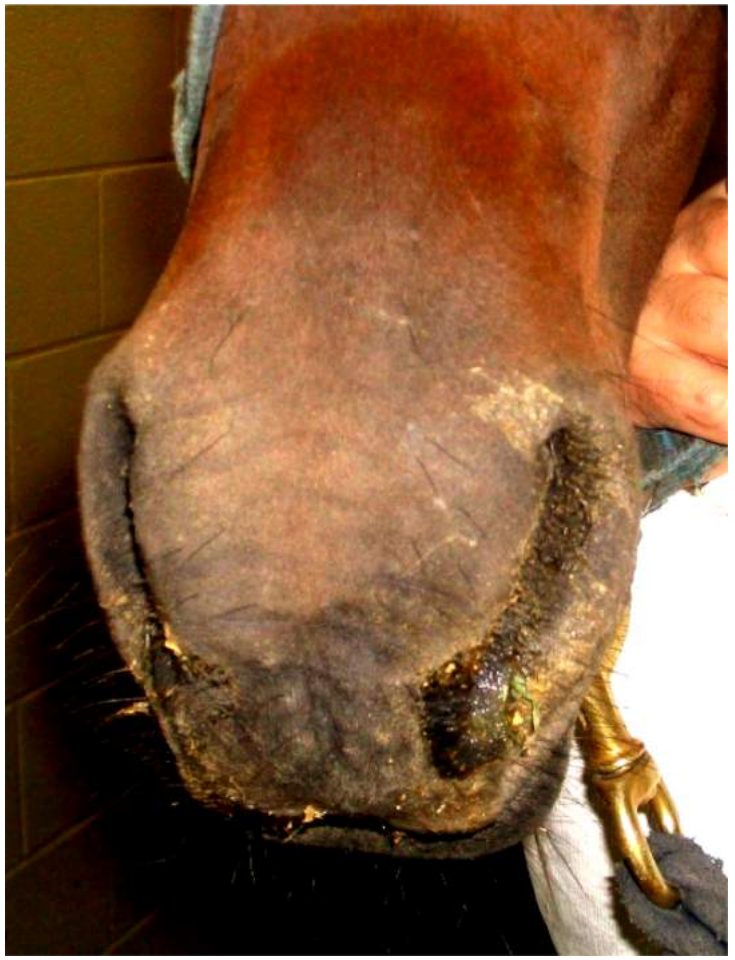
(bird)：
H5N1
…
(Human)：
H1N1
H7N9…

Equine influenza virus (EIV) infection pattern diagram



Clinical Symptoms

- ✓ fever
- ✓ frequent dry cough
- ✓ running nose(clear-thick)
- ✓ depression, muscle tenseness and soreness
- ✓ reluctant to eat or drink
- ✓ highly contagious. Can infect many horses in a short time
- ✓ infect horses of any age



History of the Equine Influenza

1299 Greece Europe
1872 U.S. equine influenza pandemic 90% of horses were sick
1956 Prague
1963 Miami
1992 Hong Kong
1993 China
2007 Australia, Japan, China, Mongolia
2012 French FEI race (vaccinated horses)

2 subtypes (Subtype):

- Equine Influenza 1 (H7N7)

The virus was first isolated in 1956

No reports after 1980

- Equine Influenza 2 (H3N8)

The virus was first isolated in 1963 and it has been circulating eversince

Diagnostic methods recommended by the World Organization for Animal Health (OIE)

OIE Terrestrial Manual 2019, Chapter 3.5.7. Equine Influenza

Method	Usage					
	Population free from EIV	No individual infection before transfer	Contribute to disease eradication strategies	Clinical case confirmation	Epidemic monitoring and epidemiological investigation	Individual or herd immunity status
Pathogen identification[1]						
Virus Isolation	-	+	-	++	-	-
RT-PCR	-	+++	+++	+++	+++	-
Antigen capture ELISA	-	++	++	+++	++	-
Immune response test						
Hemagglutination inhibition test (HI)	++	++[a]	-	+++[a]	+++	++
unidirectional radiation hemolysis test (SRH)	++	+[a]	-	+++[a]	+++	+++
ELISA	++	+	++[b]	+[a]	+++	+

Symbol: +++ means recommended method; ++ means applicable method; + means available under certain conditions, but cost, reliability, or other factors severely limit the application of this method; – means not suitable for this purpose.

1: It is recommended to use multiple antigen identification methods on the same clinical sample.

After being immunized with the vaccine of a Unidirectional radiation hemolysis test, it is used to detect the infected animals in the vaccinated group (detection of infection in vaccnation in vaccinated animals, DIVA)

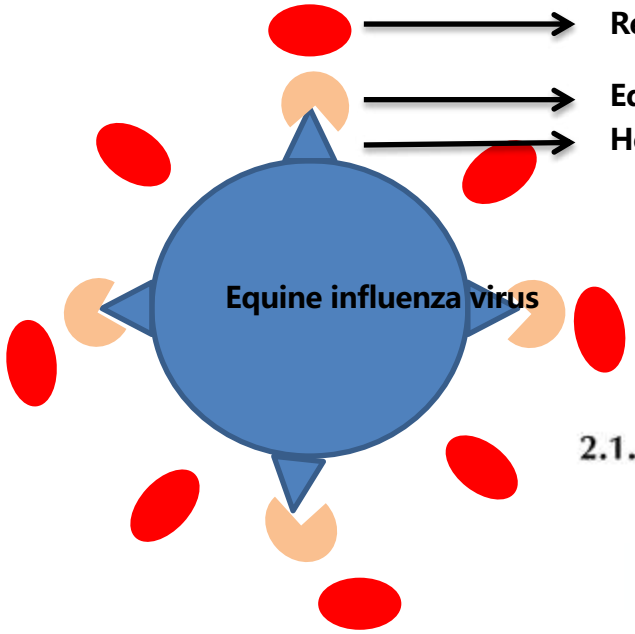
Overview of various diagnostic methods

Method	Material source	Rationale	Experiment	Note
Pathogen isolation	Nasal swab	Virus amplification	Inoculate chicken embryos and MDCK cells	Routine test, gold standard
Pathogen (nucleic acid) detection		Detection of nucleic acids (specific sequences in M and HA genes)	RT-PCR、RT-LAMP、fluorescent RT-PCR	regular RT-PCR and fluorescent RT-PCR
antigen capture ELISA		NP protein monoclonal antibody captures pathogens	AC-ELISA	Simple operation, no commercial test kits for horses
HI（Hemagglutination inhibition）	Serum	Positive serum inhibits the hemagglutination of the virus	HI that needs more pre-processing	gold standard
SRH（Unidirectional radiation hemolysis test）		Positive serum neutralizes with virus	Traditional complement fixation test	Gold standard; live virus is required, not easy to detect in large quantities
ELISA (indirect method))		Specific antibody recognition	Use NP, HA protein to establish indirect ELISA; C-ELISA	Easy to operate, no commercial kits for horses yet

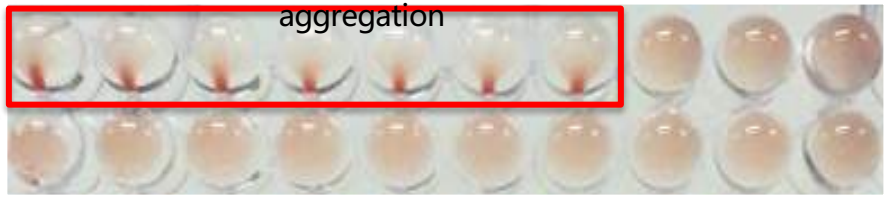
Detection technology for Equine influenza series established by our laboratory

Product name	Detection Method	self developed test kits
antibody detection	Hemagglutination inhibition test (HI)	H3N8 subtype equine influenza HI test antigen and negative-positive antibody
	ELISA	Equine influenza cELISA antibody detection kit
antigen detection	Virus isolation and identification	laboratory method
	HA	laboratory method
	ELISA	Equine influenza AC-ELISA antigen detection kit
nucleic acid detection	common RT-PCR	Equine Influenza Virus RT-PCR Detection Kit
	fluorescent RT-PCR	Equine influenza virus fluorescent RT-PCR detection kit (probe method)
	Isothermal amplification	Equine influenza virus rapid isothermal amplification kit (recombinant enzyme method)

HA/HI test-traditional standard method for antibody detection



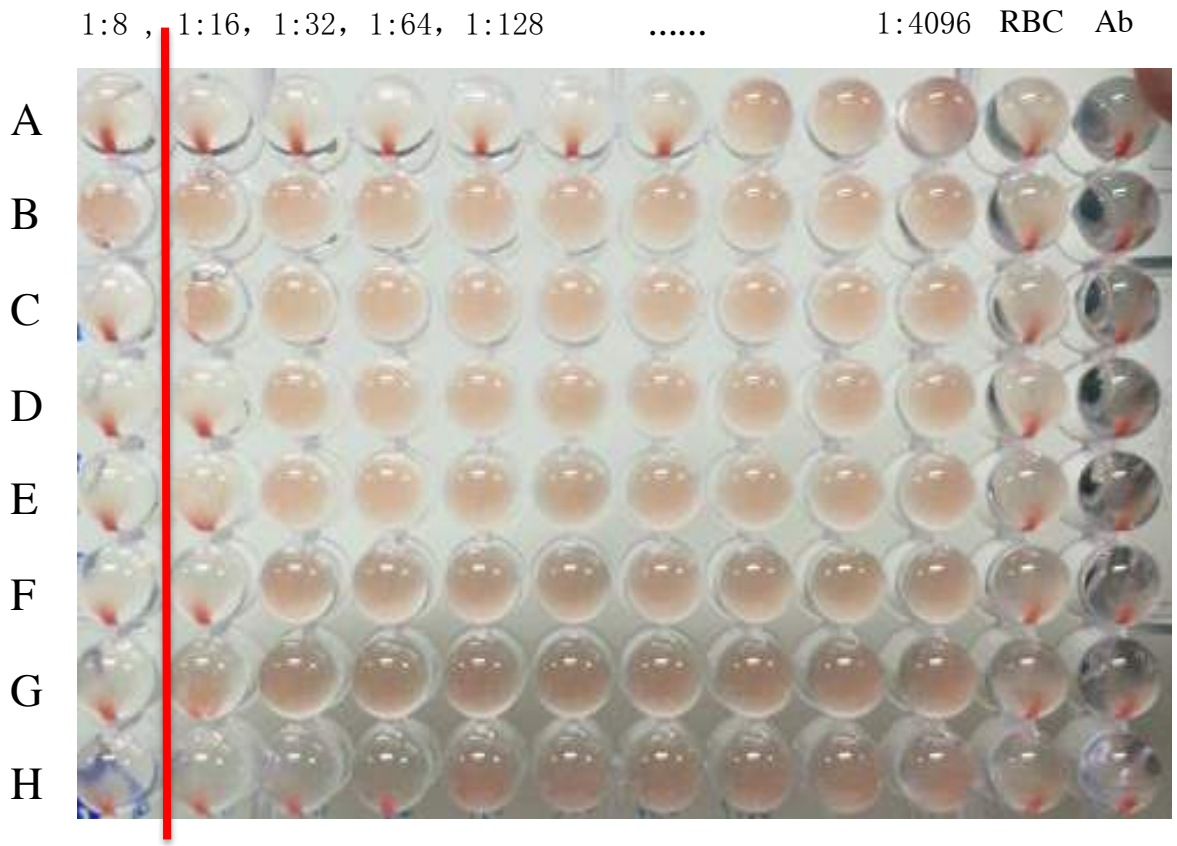
The more potent the hemagglutination inhibition → **The higher the antibody content per unit volume of serum sample**

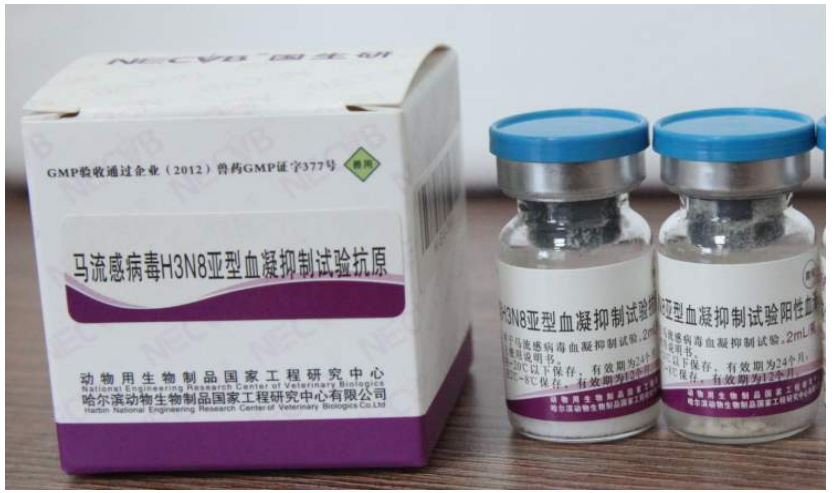


2.1. Haemagglutination inhibition test

The antigen is first treated with Tween 80/ether in order to increase the sensitivity of the test, particularly for H3N8 viruses. The HI test can also be performed without ether treatment although at reduced sensitivity. The test is best done in microtitre plates using the appropriate dilution equipment. A macrotest may be used, for which antigen is diluted to a final HA titre of 1/8 per well and the volumes for PBS, sera and antigen are 0.5 ml. Sera are pretreated to remove nonspecific haemagglutinins, then inactivated at 56°C for 30 minutes. Pretreatments include the use of one of the following: (a) kaolin and RBCs absorption, not recommended for H7N7 HI, (b) potassium periodate, or (c) *Vibrio cholerae* receptor-destroying enzyme. Potassium periodate or *V. cholerae* receptor-destroying enzyme is the treatment of

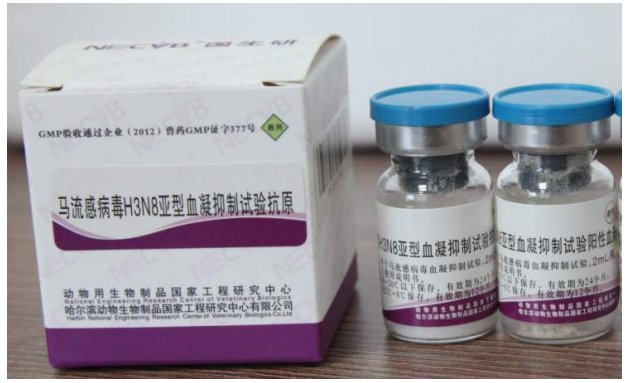
Equine influenza virus hemagglutination inhibition (HI) test antigen and negative and positive sera





A：Positive serum control；B：Negative serum control；C~H：Different test samples；
RBC：red blood cell control；Ab：antibody control

Application of EIV HI Detection



1.Identification of pathogen typing

Influenza virus HA antibody has type specificity, equine influenza HI positive sera can be used for the identification of equine influenza isolates

2.Identification of equine influenza infection in non-immune animals

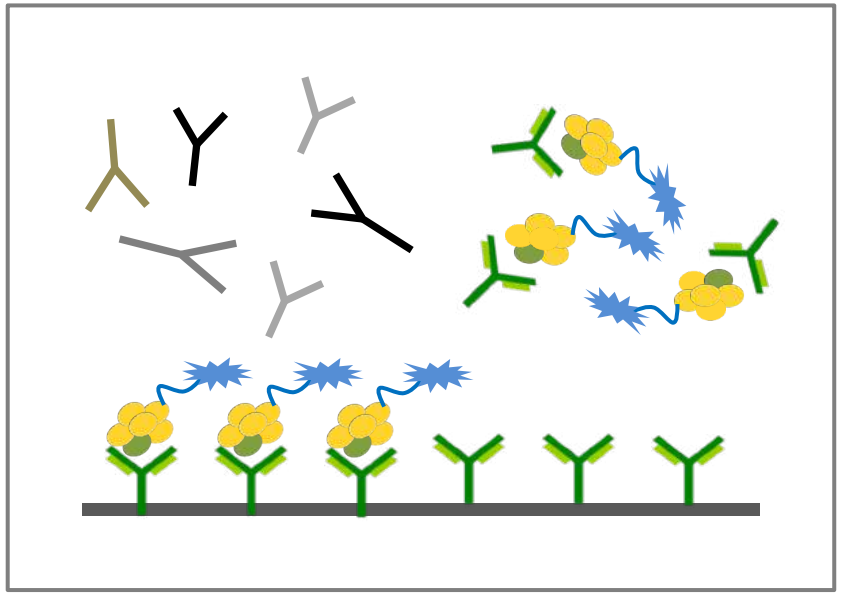
Collect double sera (10 days apart) from the equine animal to be tested for HI test. If the HI titer of the second serum is higher than that of the first serum, it indicates that it is in the infectious period.

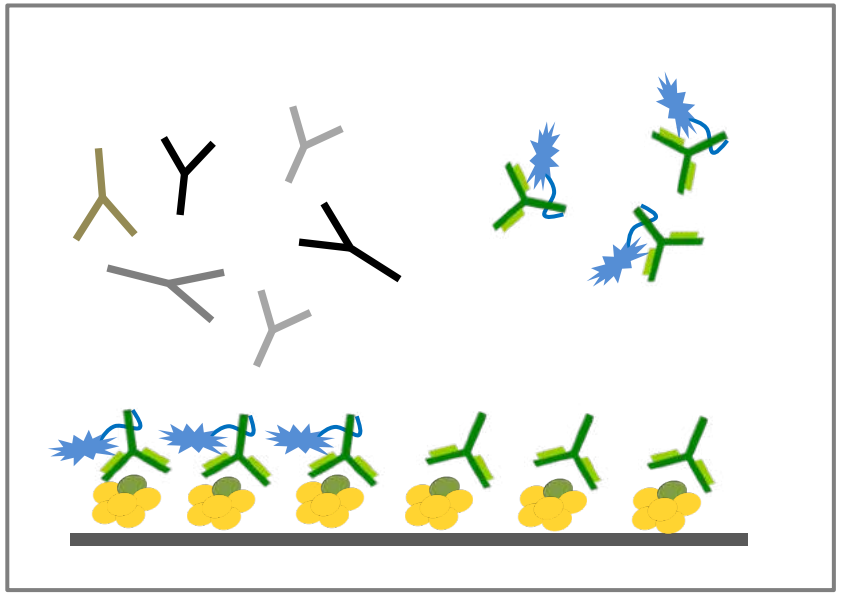
3.Evaluation of Immune Antibody Level

It is generally believed that equine influenza vaccines can be effective against EIV when the vaccinated horses' HI titer is not less than 1:64. The higher the HI titer, the higher the resistance of equine animals to EIV.

Equine influenza cELISA antibody detection method

⟺ Influenza A virus antibody test





Test Steps	Similar kit 1	Similar kit 2	HVRI
Mix the sample to be tested with the enzyme-labeled secondary antibody	Incubate at 37°C for 1h		
Wash plate	Yes		
Color development time	Avoid light and develop color for 10min		
Reading	OD450		
Result Determination	Competition rate ≤45% is positive	Inhibition rate>45% is positive	Competition rate ≥45% is positive

Equine influenza cELISA antibody detection method

reaction condition	coated monoclonal antibody	Sc-1	Sc2	Sc-1	Sc-2
OD Value	Test steps	two step approach	two step approach	one step approach	one step appro
	PC undiluted	0.058	0.048	0.058	0.057
	NC average value	1.768	1.738	1.708	1.66
inhibition rate	#1 1x	100	100	100	100
	#1 2x	95.67	94.91	95.15	96.51
	#1 4x	76.2	82.66	80.48	85.09
	#1 8x	46.37	54.62	61.39	70.56
	#1 16x	18.13	26.75	27.88	42.67

completed in 30 minutes

lab cELISA	HI test +	HI test −
+	77	6
−	9	27

similar test kit 1	HI test +	HI test −
+	63	3
−	23	30

Conclusion 1: The detection rate of cELISA is higher than similar kits

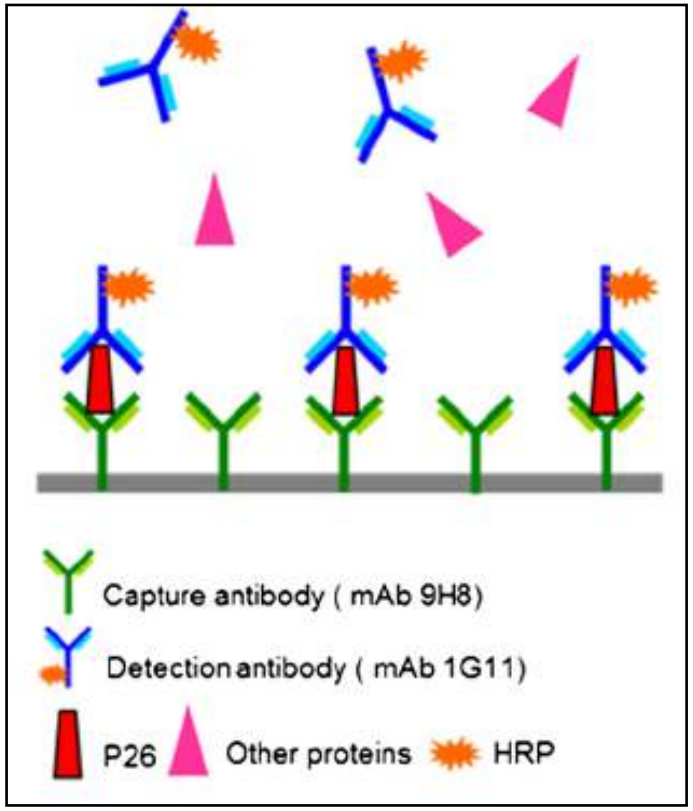
method		similar test kits 1	HI test
lab cELISA	Negative coincidence rate	72.73%	81.82%
	Positive coincidence rate	100.00%	89.53%
Ingina Reagent test kit	Negative coincidence rate	-	90.91%
	Positive coincidence rate	-	73.26%

Conclusion 2: The positive coincidence rate of cELISA and HI test is better than similar kits

Equine influenza AC-ELISA antigen detection method

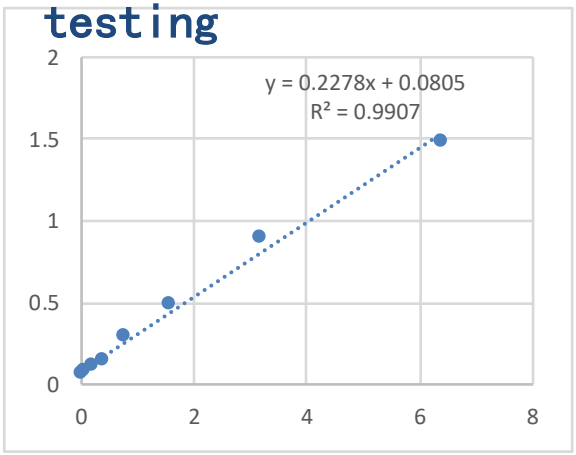
⟺

Influenza A virus antigen detection

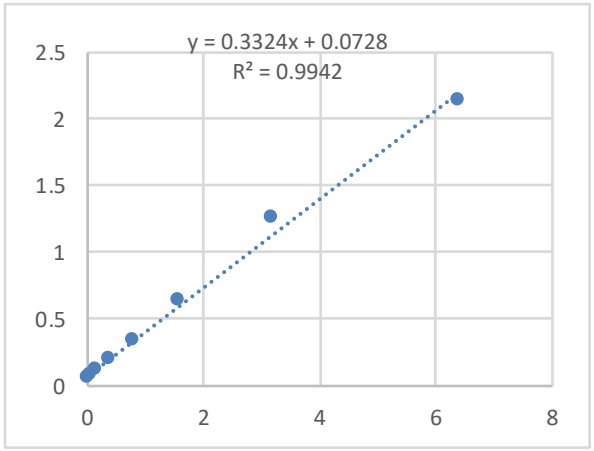


test steps	similar test kits	HVRI
samples to e tested100ul	incubate at room temperature for 25min	incubate at 37 celsius for 30min
wash plate	no	no
Whether to discard the liquid in the well	no	yes
Enzyme-labeled secondary antibody	incubate at room temperature for 25min	incubate at 37 celsius for 30min
wash plate	yes	yes
color development time	color developed at room temperature for10min	Avoid light for 5min
reading	OD450	OD450
result determination	Positive is when OD value>0.22	Positive is when OD value>0.12
total test time	60min	65min

1. Sensitivity testing



AC-ELISA



overseas similar test kits

The sensitivity of AC-ELISA is 2 times higher than that of similar kits.

2. specificity testing

samples to be tested	OD value	result
negative control of overseas products	0.050	negative
EIAV	0.068	negative
EHV-1	0.069	negative
EHV-4	0.062	negative
EAV	0.062	negative

AC-ELISA has strong specificity and all other viruses are negative

3. detecting negative control

samples to be tested\method (threshhold value)	HVRI (OD＞0.12)	similar kits (OD＜0.20)
negative control of similar kits	0.050	0.051
PBST	0.063	0.12
Swab diluent	0.065	0.13
Antigen antibody diluent	0.074	0.16
negative serum1	0.057	0.124
negative serum2	0.056	0.132
negative serum3	0.059	0.12

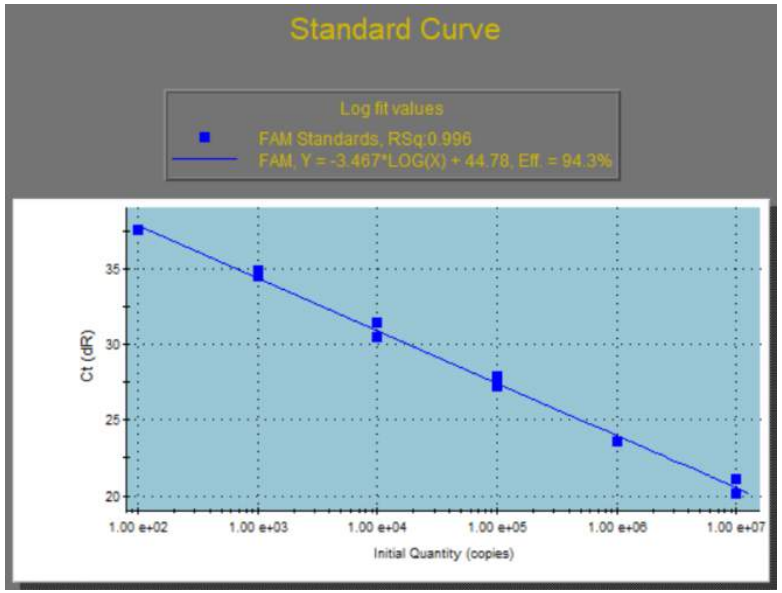
The negative result of AC-ELISA test has lower OD value, indicating good specificity

4. Detection of clinical nasal swab samples

Using AC-ELISA and foreign kits to test 52 clinical nasal swab samples, 3 positive samples and 49 negative samples were detected. The positive and negative coincidence rates of this method were both 100%.

Fluorescence RT-PCR detection method

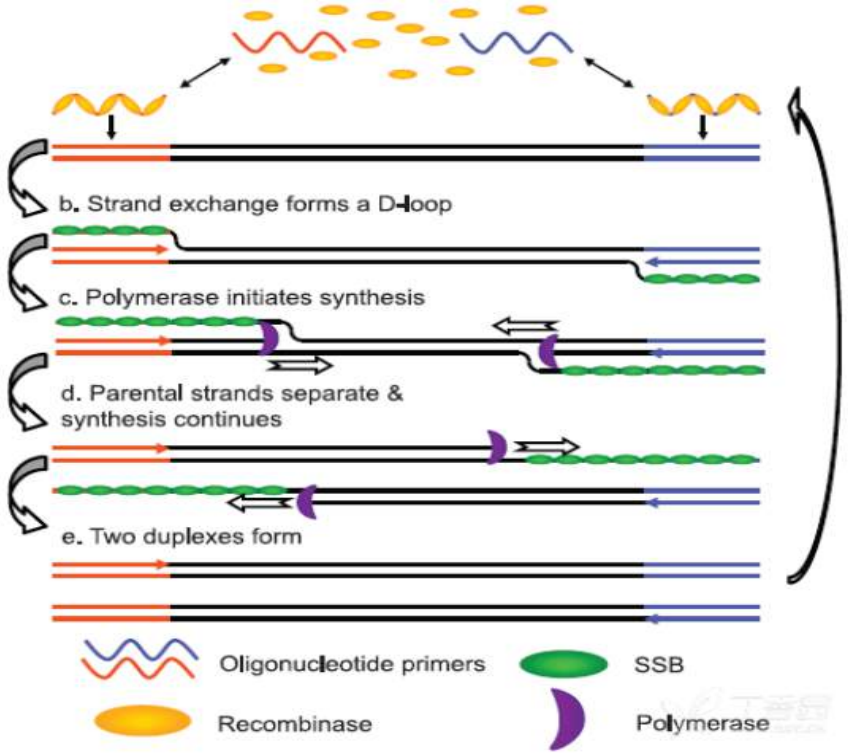
test method	OIE approach	laboratory
target gene	M gene	NP gene
Amplification efficiency	89%	94.3%
Positive detection rate	51.92%	53.85%



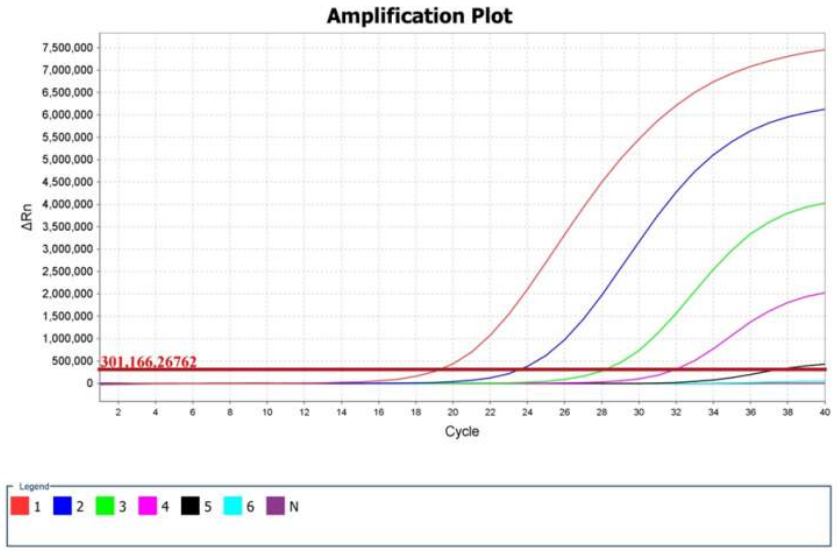
Amplification efficiency : 94.3%

The EIV fluorescent RT-PCR method established by our team is better than the detection method recommended by OIE

EIV recombinase mediated isothermal nucleic acid amplification （RT-RAA）



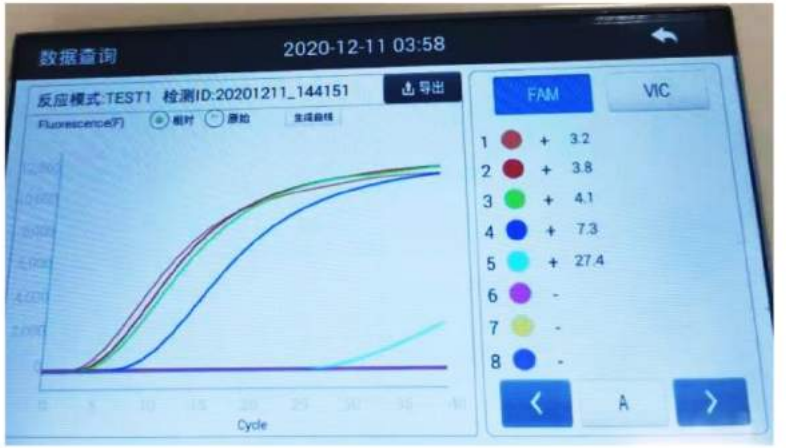
（recombinase aided amplification, RAA）



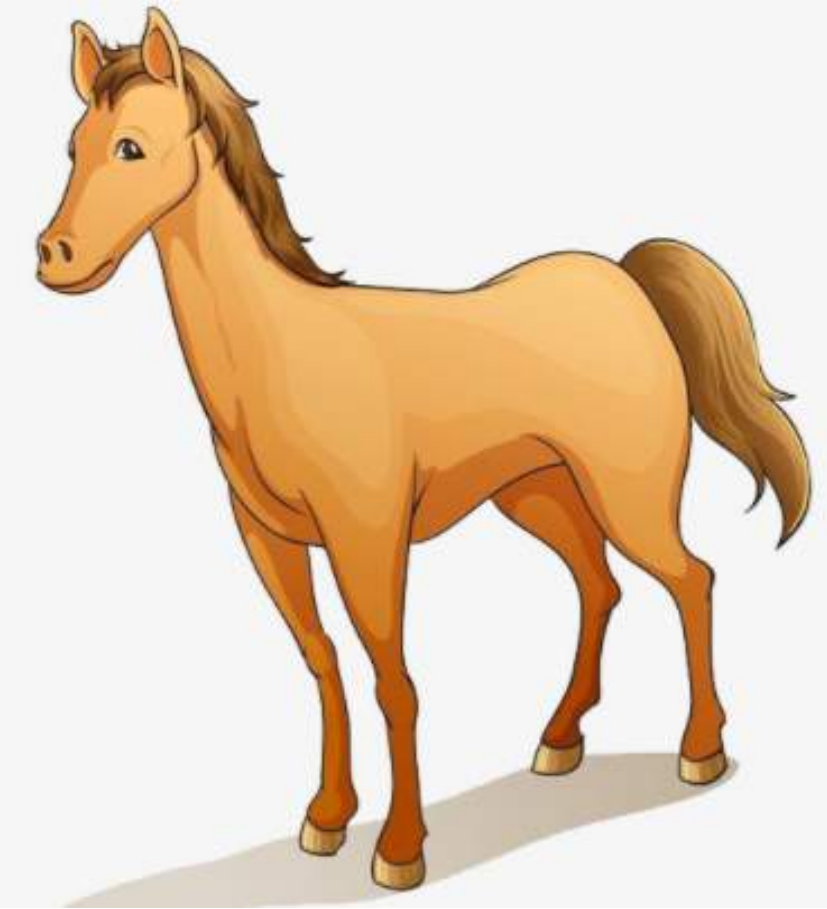
样本名称	目的基因	Ct值
1	稀释10^{-1}倍	19.256
2	稀释10^{-2}倍	23.577
3	稀释10^{-3}倍	28.169
4	稀释10^{-4}倍	31.913
5	稀释10^{-5}倍	37.406
6	稀释10^{-6}倍	-
N	阴性	-

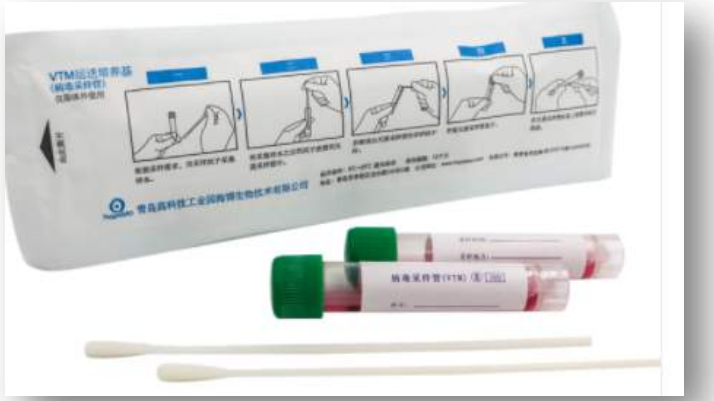
EIV isothermal amplification method has the same sensitivity as fluorescence method, and the detection time is shorter, only 20 minutes.

Instruments and consumables for recombinase-mediated amplification technology



How to carry out EIV testing clinically?





◎ Equine influenza virus nucleic acid detection (fluorescence RT-PCR), if it is positive, further virus isolation and identification will be performed for confirmation;

◎Equine influenza antigen test (AC-ELISA) can be used for preliminary diagnosis.

◎Equine influenza virus antibody test can help to understand the animal's infection history;

◎The antibody titer increased by more than 4 HI within 2 weeks, which proves that the animal is sick.

Fresh and complete samples are the key to successful testing!

Lab	Test	Samples	Amounts
HRVI	HI	Serum	84
	SRH	Serum	84
	Real-time PCR	Nasal swabs	20
IEC	HI	Serum	154
	SRH	Serum	154

The Harbin Veterinary Research Institute and the Irish Equine Centre (IEC), the OIE Equine Influenza Reference Laboratory, established the TWINNING project to conduct a comparison of standardized diagnostic techniques for

At the end of the Twinning project the Irish Equine Centre assisted Professor Wang in the organization of a workshop at HVRI with delegates from other laboratories in the region where the role of an OIE Reference Laboratory and the support available from HVRI in relation to the diagnosis and control of equine influenza was explained. Professor Wang informed me since that meeting HRVI has provided support to Kazakhstan in equine infectious diseases diagnosis and helped S. Seifullin Kazakh Agro Technical University to build a biological laboratory. He has also had many communications with the Hong Kong government and the Hong Kong Jockey Club.

Professor Wang is a respected researcher of international repute and his highly skilled team including Associate Professors Zhe Hu and Wei Guo, have the potential to make a significant contribution to equine influenza control. Provided HVRI fulfils the OIE Terms of Reference (http://www.oie.int/scientific-expertise/reference-laboratories/guidelines-for-applicants/), I have no hesitation in recommending that the OIE designate Professor Wang an OIE expert for equine influenza and that his laboratory at HVRI be designated an OIE Reference Laboratory.

Yours Sincerely,

Ann Cullinane 4th July 2018

Ann Cullinane, *MVB, PhD, MRCVS*
Head of Virology, Irish Equine Centre
Adjunct Professor, University of Limerick
Adjunct Professor, University College Dublin,
OIE expert, Equine Influenza Virus and Equine Rhinopneumonitis
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- Through multiple comparison tests, the test results of both parties are consistent, indicating that the equine influenza diagnostic method in this laboratory meets the OIE requirements.
- Ann Cullinane, Chief Scientist of OIE Equine Influenza and Equine Rhinopneumonia, recognized that our laboratory has the capability of an OIE reference laboratory.

Thank You

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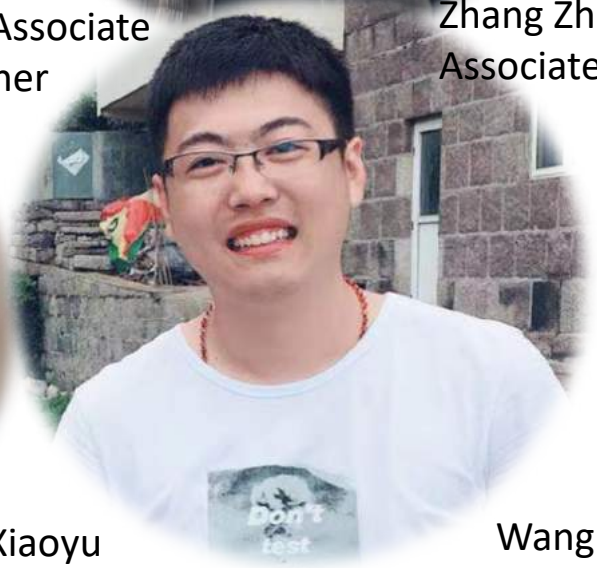

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