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Utilizing Haemagglutination Property of Rabies Virus for Detecting the Pathogen and Checking its Destabilization

Pham Hong Son*

Hue University Member University of Agriculture and Forestry, 102, Phung Hung, Hue city, Thua Thien Hue province, Vietnam.

***Correspondence**
Pham Hong Son
Hue University Member University of Agriculture and Forestry, 102, Phung Hung, Hue city, Thua Thien Hue province, Vietnam.
E-mail address: phongson@hueuni.edu.vn

Abstract

The haemagglutination property of viruses is useful for titration and condensation of virions of their own, as well as for titration of antibodies specific to them, but detection of the pathogens in clinical samples. However, on the base of the haemagglutination (HA) and haemagglutination inhibition (HI) titration techniques a derivative technique was recently contrived for detection of haemagglutinating viruses in animals called the method of Assay of Shifting of Standardized Direct Haemagglutination Inhibition, or SSDHI. We herein report on the application of these HA-based techniques in cases of rabies virus. With pictorial evidence in detecting the microbe in saliva fluids of furious and healthy dogs in Central Vietnam for disease diagnosis and epidemiological investigation, some results on detection of rabies virus in clinical samples, criteria of discrimination of "coinfections" with other haemagglutinating viruses and possible "contaminations" with specific antibodies or their analogues in clinical samples were discussed. The techniques did also help prove that air-dried virions of rabies virus from clinical samples can sustain their integrity at room temperature at least for two days which implies possibility of the pathogen's transmission through aerosols.

KEYWORDS
HI, Laboratory diagnosis, Rabies, SSDHI, Virion integrity

INTRODUCTION

Diagnosis of infectious diseases and investigation of infective pathogens in series of cases require laboratory tests, as for the case of rabies (OIE, 2019) has stated that the only way to undertake a reliable diagnosis of rabies is to identify the virus or some of its specific components using laboratory tests. There are many methods for verifying clinical and pathological observations, including serological, haematological, biological, and molecular biology-based techniques available worldwide with different levels of reliability, precision, tediousness, and harmfulness. Indeed, those methods that aim to finding infective germs' structural components, such as their antigens (specific proteins) or nucleic acids, are of high importance, implying the presence/absence of the pathogens themselves. However, in acid nucleic analyses, in which amplification processes of target molecules take place, often a lot of plastic tools or equipment parts must be disposed to avoid cross contamination. These techniques, as well as the most of antigen detection ones, also cannot assure that the detected pathogens are in intact form of their own, as the targets are molecules or their portions/domains (as epitopes, or determinant groups), meanwhile sometimes we have to survey the presence of intact viral particles (an inevitable property implying viability of viruses) in the environments. Since that, to respond to the demands of confirmative diagnosis and epidemiological surveys of the viral infection, in considering the strengths and weaknesses of all already available diagnostic tests and based on the haemagglutination property of some viruses we have established a new protocol called Assay of Shifting of Standardized Direct Haemagglutination Inhibition (SSDHI). This technique can be partially automated using microtitration 96-well plates, which were designed previously for implementing haemagglutination (HA) (Hirst, 1941) and haemagglutination inhibition (HI) reactions (Clarke and Casals, 1958; Sever, 1962; Cottral, 1989). We herein introduce some results we have obtained using SSDHI in combination with HA in confirming the presence of the rabies virus virions in some furious rabid dogs, surveying rabies virus prevalence, checking virions' destabilization/sustainability, and by the way, show possible oddities that may appear with implementing SSDHI protocols and their amelioration measures.

MATERIALS AND METHODS

Material preparation

Serum samples of dogs were collected, preserved at minus 10 °C for cases of delayed use, and tested with HI for specific antibody titres.

Saliva samples were collected individually from caged furious dogs for confirmative diagnosis tests of rabidity cases and from clinically healthy dogs reared in local households for infection surveillance and used 25 µL volumes of them for each SSDHI re-

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action.

As sources of the standard viral antigen for implementation of HA, HI, and SSDHI, commercially available Rabigen® Mono (Virbac, France) lyophilized live-attenuated rabies vaccine was used. It was subjected to haemagglutination test with chicken red blood cell (RBC) 1% suspension to determine the viral titres, and on the base of the test's results, the vaccine was diluted with saline solution to obtain 2 log_2 HA viral suspension, which then was assayed again with four wells of microtitration plate for confirmation of the virus titre, adjusted for the right concentration if necessary, and used for HI and SSDHI reactions or aliquoted into small (1 mL) tubes and kept at minus 10 °C for delayed use.

To have 1% RBC suspension, chicken blood samples, usually from a slaughterhouse, were collected in test tubes containing sterile 3% tri-sodium acetate solution as anti-coagulant (one tenth volume). To them physiological saline solution (0.9% NaCl) was then added, mixed and the tubes were centrifuged at 200 ×g for 2 minutes for harvesting the pellets. At the end of the 3rd time of spinning, an amount of 0.5 mL of the RBCs at the bottom of the tubes were sucked out into a pipette tip and mixed with some volume of saline solution in a graduated cup. Additional amounts of saline solution were added to reach a total of 50 mL to have 1% RBC suspension while mixing.

To have standardized 4 HA virus suspension, the reaction of HA (Hirst, 1941) was performed in a 12-well row of a 96-well plate, by making serial dilution of virus-containing material in the first 11 wells with constant volumes of 25 μL saline solution while leaving the 12th well with no virus to serve as the indicator of initiation moment of reading the reaction afterwards, and then adding 25 μL of the 1% RBC suspension to each of the 12 wells. Titre reading was done when the negative control in the 12th well of the rows showed that all the red cells settled into the well's bottom center forming a clear red point.

To have 4 log_2 HI sera, blood samples from dogs were let to coagulate and the liquids were checked for antibody titres with HI reactions (Clarke and Casals, 1958; Sever, 1962; Cottral, 1989) with the prepared 1% RBC suspension and the above-obtained 2 log_2 HA virus suspension. And for the further application, based on the results of HI, the positive serum samples were diluted in saline solution and mixed to make initial 4 log_2 HI specific serum. This was then checked by HI reactions with five levels of replacement of 0%, 10%, 20%, 30%, and 40% of saline using a "saline ten-percent replacement series" protocol of serum dilution, as shown in Table 1, to determine the concentration range of specific antibodies in the serum of 4 log_2 HI titre. As a rule, it ranges from 16 HI units (4 log_2 HI) to nearly 32 HI units (nearly but not enough to demonstrate 5 log_2 HI). After performing five rows of HI reactions, the row with the highest replacement percentage that showed the same appearance (3 log_2 HI reaction) as did the first (0% replacement) row was determined. Based on the replacement percentage value of this row, a suitable amount of physiological saline was added to the initial 4 log_2 HI serum to make standardized 16 HI unit rabies-specific serum (with 16 - ~16,66 HI unit range concentration) ready for implementing SSDHI or aliquoted and preserved at minus 10 °C for delayed use.

Sampling

For diagnostic tests of rabies, saliva fluids were taken during furiousness of rabid-suspected dogs (Figure 1), the samples were individually kept on ice and subjected to SSDHI tests. The collection of the samples was performed by insertion of pinches of clean dry cotton fixed on forceps into oral cavities of confined dogs until the cotton pieces became saturated with saliva. Each of the cotton pieces was then inserted into a small polyethylene bag and squeezed by pressing with fingers from outer side to flow the saliva into a bottom corner of the bag, and transferred with a pipette into an Eppendorf tube ready for SSDHI testing or, in cases of delayed assays, marked and kept in a freezer compartment at minus 10 °C until the assay.

For surveillance, we collected samples of dogs' saliva from September of a year to February of the next year in the period of 2017-2021. It was at least 6 months after nationally recommended annual vaccination campaigns in March and April, so there was a percentage of unvaccinated individuals amongst the sampled dogs. From each dog, both saliva for antigens and blood for serum were collected at the same time of confinement (usually the animals were handled by the owners). For serum collection, after a saliva-saturated cotton piece was taken out and kept in a bag on ice, the dog was bled from a vein with a needle fixed on a syringe. About 2-3 mL of blood were drawn out from each dog each time and let coagulate at room temperatures. Each sample of the sera was transferred into an Eppendorf tube and subjected to testing or kept frozen in cases of delayed testing. The sera were then tested with HI, adjusted for 16 HI unit antibody concentrations and transferred into Eppendorf tubes to be kept at minus 10 °C for further use.

For checking the capacity of sustaining intact of rabies virus particles (virion sustainability/disintegration) when exposed to air-drying, daily a pair of filter paper discs were impregnated each with 50 μL of 3 log_2 HA rabies virus-positive saliva and air-dried in a biohazard chamber at room temperatures. At the end of the exposure of the 10th day each of the virus-carrying paper discs was placed in an Eppendorf tube. To each of the tubes 100 μL of distilled water were added, and the tubes were kept at 4 °C for 24 hours, vortexed to reconstitute the viral suspensions. One of each pair of the paper discs was treated with 20 μL chloroform, mixed well and let stand. Viral watery suspension beneath

Table 1. Steps supposed to prepare a "saline ten-percent replacement series" of 3 log_2 HI sera

The 1st wells with 5 dilution rows' tags	Step 1: Deliver initial 4log_2 HI serum (μL)	Add saline (μL) and mix to dilute serum	Step 2: Transfer serum (μL) to create 3log_2 HI sera	Step 3: Transfer serum (μL) to create 10% series	Step 4: Fulfill 10% series: add saline (μL)	Outcome: Saline replacement percentages	Final volume (μL) ready for the 1st wells for checking 3 log_2 HI reactions	Working HI plates: The volume of the 1st wells (μL)
r1	100	100	100, to r2	-	-	0%	100	25
r2	-	-	100, from r1	10, to r5	10	10%	100	25
r3	100	100	100, to r4	20, to r5	20	20%	100	25
r4	-	-	100, from r3	30, to r5	30	30%	100	25
r5	-	-	-	60, from r2,3,4	40	40%	100	25

HI sera obtained from a stock initial 4 log_2 HI serum in the first wells of 5 rows to carry out HI reactions to determine possible ratios of saline solution to be replaced in the stock initial 4 log_2 HI sera for decreasing the antibodies' concentrations to the lowest levels of titer units within the same titer logarithmic category of 4 log_2 HI to maximize the sensitivity of SSDHI

of the chloroform layer was drawn by pipette tips from each tube. Meanwhile, the other ones of the pairs of viral suspensions were left untreated chloroform. The presence of rabies virus in the suspensions of all the 10 consecutive days of air-dry exposure, either with or without chloroform treatment, was checked with both SSDHI and HA assays.

SSDHI

Each reaction of SSDHI, the basis of which was introduced previously somewhere (Pham and Nguyen, 2017) for every saliva sample was performed in an 8-well row of a 96-well plate, thus, a maximum of 11 samples can be tested in parallel with one row of the standard 16 HI unit antiserum reaction serving as the negative control for reading the tested samples on each plate. At first, a 25 µL volume of saline was pipetted into each of the 7 wells from the second one of each rows, leaving the first (leftmost) wells blank. To each of these first (blank) wells a 25 µL volume of each of the to-be-tested saliva samples was then added (up to 11 samples in each micro titration plate), and 25 µL of saline was added to the first well of one row (usually the last row) for negative control (e.g., standardized 4 log_2 HI antiserum control). Marking the rows in accordance with sample specifications is preferable. Then, to each of all the first (leftmost) wells of all the rows a 25 µL volume of standardized 16 HI unit anti-rabies serum was added in expecting reactions between the specific antibodies and viral antigens possibly present in the samples. By pipetting, the contents of the first wells were mixed, and then transferred by 25 µL each time from one well into the next well of the same row until the 7th wells, and from each of these 7 wells, 25 µL of the mixtures were discarded to make two-fold serial dilution in the rows of seven wells, leaving the last (8th) well with saline alone to serve as standard indicators for determining the moment of reading the reactions. A volume of 25 µL of the 2 log_2 HA virus suspension was added into each of all wells of each row, except the last (8th) wells, leaving these rightmost wells with saline alone again. After about 10 minutes, 25 µL of the 1% RBC suspension was added into each of all of the wells. The results were read after 15-30 minutes when in the last wells of the rows the red cells settled completely forming red points, meanwhile the same phenomenon of RBC sedimentation was seen also in the four leftmost wells of the standard 16 HI unit serum (negative control) row (showing 4 log_2 HI reactions).

Statistical analysis

Ratios were compared with chi-square analysis with the aid of Microsoft Excel software, the two ratios would be different if the probability is $p<0.05$.

RESULTS

Case study: confirmation for the presence of rabies virus in dogs with symptoms of furious rabidity

This is information we obtained from analyzing saliva samples collected from dogs with symptoms of furious rabidity (Figure 1).

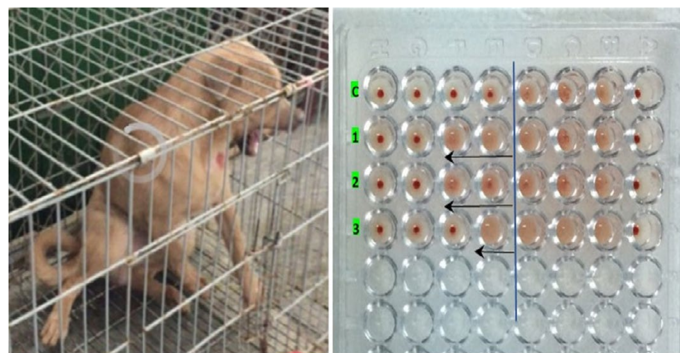

Figure 1. A dog with symptoms of furious rabidity (left picture), which was confirmed with SSDHI to have rabies virus in the saliva (row "1" in the right picture) with other two tests of saliva fluids collected from other two rabid dogs (the 3rd and 4th rows). The sample reactions show shifting two-, two-, and one-well leftwards in comparison with the standardized 16 HI unit antibody serum reaction in the 1st row (designated as "C" for "control") indicating SSDHI 2+, 2+, and 1+, respectively.

Testing with SSDHI for diagnosis of three furious dogs showed that in all the three samples of saliva fluids exhibited leftward shifting of HI reactions in comparison with the negative control (C) where no sample was added. The levels of shifting of the three samples were different, with respective two-, two-, and one-well leftwards. This means in all the saliva samples from three furious dogs clinically suspected as rabid there were factors that could "neutralize" the inhibition effects of anti-rabies antibodies in the standardized 4 log_2 HI serum on the virus' haemagglutination.

Application of SSDHI in a rabies epidemiological investigation

The expression of exemplar four plates of performed SSDHI tests with 44 representative saliva samples in the mentioned epidemiological investigation is demonstrated in Figure 2.

Testing with saliva samples (Table 1) of 1723 dogs (all with no symptoms of sickness) reared in lowland areas of Thua Thien Hue province and in mountainous communes of Quang Binh province collected from 2017 to 2021 with such SSDHI showed that the rate of positive cases of infection with rabies was 2.84% with no clearly significant difference between areas (P = 0.08). This indicates that infected dogs shed rabies virus in saliva fluids even when no signs of the disease have appeared.

Determination of destabilization of rabies virions

Suspensions from SSDHI-positive samples from air-dried virus-impregnated filter paper discs without and with chloroform treatment after different periods (from day 0 to day 10) of exposure to room temperatures (25-28 °C) were subjected to HA and SSDHI tests (Figure 3). For chloroform-untreated sample, both the SSDHI and HA reactions showed positive at day 0 (virus suspension reconstitution control), day 1 and day 2 of exposure to room temperatures in sterile and dry conditions, and then became negative at all the later days. With SSDHI all the samples from the first three days of exposure were at level 1+ (the left picture). Meanwhile, with HA the reactions were at 2 log_2 at day 0, and 1 log_2 at both days 1 and 2 of exposure (the central picture), and no more positive appearance after that. Conversely, in cases

Table 2. Outcomes of SSDHI tests with saliva samples collected from some different localities in Central Vietnam from 2017 to 2021.

Locals and years of sampling	Tested dogs	Positive	Prevalence	Chisq.test P
Thua Thien Hue (urban)	813	28	3.68%	0.085
Quang Binh (rural, mountainous)	910	19	2.07%	
Total	1723	47	2.84%	

of chloroform treatments (the right picture) the positive appearance of SSDHI reactions could not be seen in all virus suspensions from day 1, in contrast to the results from the paper discs tested prior to chloroform treatment at day 0 as the virus reconstitution control.

DISCUSSION

Usually, diagnostic tests have the aim to confirm or exclude a suspected microbe as the pathogen responsible for an illness. For rabies, as well as all the other viruses, to prove the presence of specific components of the virus is confirmative.

The principle of SSDHI demonstrated illustratively in Figure 4 helps explain why, when specific virus particles are present in a sample, the HI titre of specific 4 log_2 HI serum in the corresponding sample row of an SSDHI plate changes decreasingly, leading to "shifting" leftwards in comparison with the standard, or negative control, row as seen in the second picture of Figure 1. All viruses have one or more kinds of spikes on their virion surfaces serving as ligands to host cell surface receptors usually triggering the internalization of the virus genome into host cells. Previous researchers have found a unique G protein exposed on the surface of rabies virus serving as the major determinant of its pathogenicity and major protective antigen responsible for inducing protective immunity against rabies (Wiktor *et al.*, 1973; Cox *et al.*, 1977). Series of other observations (Pham and Nguyen, 2017) added that surface spikes of rabies virus have another important property, the capability of haemagglutinating, which allowed us implement HA and HI tests, and then create SSDHI.

In Figure 4, on the first line (in respect to the mathematical signs) the principles of HA reaction (Hirst, 1941), then, on the next line that of HI reaction (Clarke and Casals, 1958) are demonstrated, and based on these two reactions the technique of SSDHI is formulated (on the third line). In HA reactions, the formation of agglutination of RBCs is not virus-specific since many viruses in the nature can bind to animal RBCs and make them join together into tridimensional lattices, thus not allowing the cells settle to the bottom of well. For that reason, HA results give ambiguous answers with clinical samples and can be determinative only in cases of titration of already known haemagglutinating viruses. HI reactions are, on the opposite, virus-specific as the already determined kind of virus (vaccine) can be blocked only by compatible antibodies, so it helps detect the presence and determine the concentrations (titres) of antibodies specific to the virus, and has been considered as a "gold standard" for antibody detection and titration (Prittie, 2004).

Since agglutination appears only when the numbers of equivalents of RBC reactants (surface receptors) and virus surface reactants (viral haemagglutinins) are nearly the same, too highly

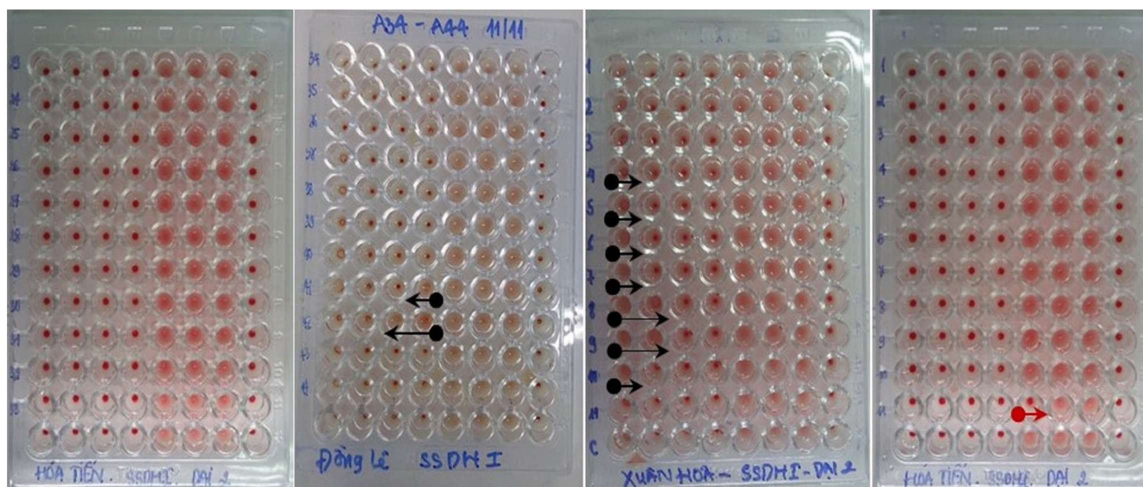

Figure 2. Four representative microtitration plates with 44 saliva samples tested with SSDHI on them: Almost all samples are negative except for two samples (#41 and #42, the rows marked with leftward arrows in the second picture) that show leftward shifting phenomena of haemagglutination inhibition titer (with respectively 1 and 2 well levels) in comparison with the standard 4 log_2 HI unit reaction in the lowest row of wells. The first picture with all negative reactions showed that normally dog saliva fluids serve as physiological solution in the reaction. In addition, we can see haemagglutination (with rightward arrows) in some initial (leftmost) wells in the third picture, and haemagglutination inhibition in an additional well (with a red rightward arrow) in the fourth picture.

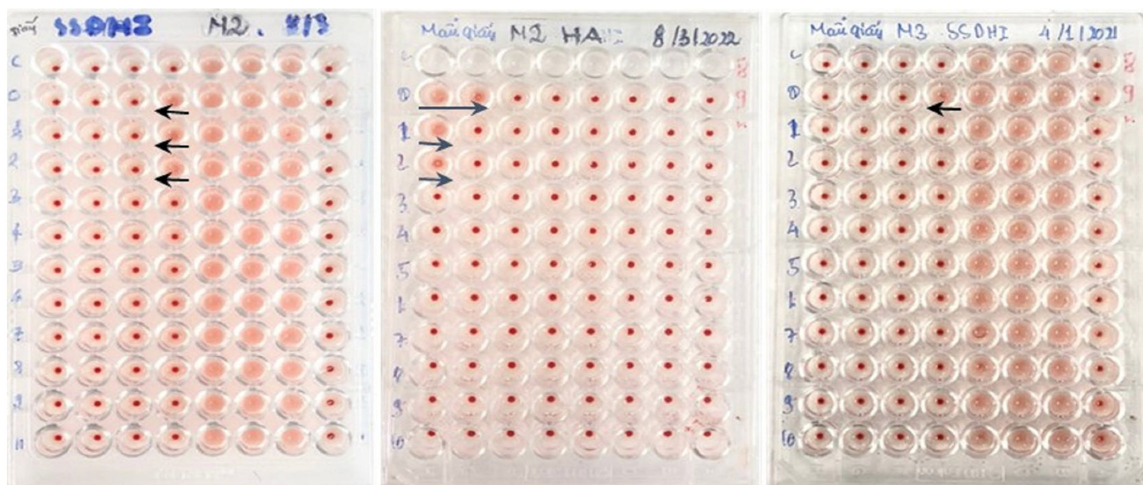

Figure 3. SSDHI and HA tests for sustainability of rabies virus from saliva fluids of positive dogs showed that at room temperatures in sterile conditions the virions could sustain intact in two days, detectable with both SSDHI (in the first pictures: marked with arrows indicating shifting leftwards) and HA (in the second picture: marked with arrows indicating shifting rightwards); and no SSDHI-positive reactions remained after chloroform treatment (in the third picture: samples of all days of treatment showed negative, except for virus suspension reconstitution control (O) with no chloroform treatment, marked with an leftward arrow). Note that every SSDHI plate shown here has two control reactions: one negative control for standardized 4 log_2 HI anti-sera (C) and, next, another control for double-diluted virus suspension serving as reconstitution control (0).

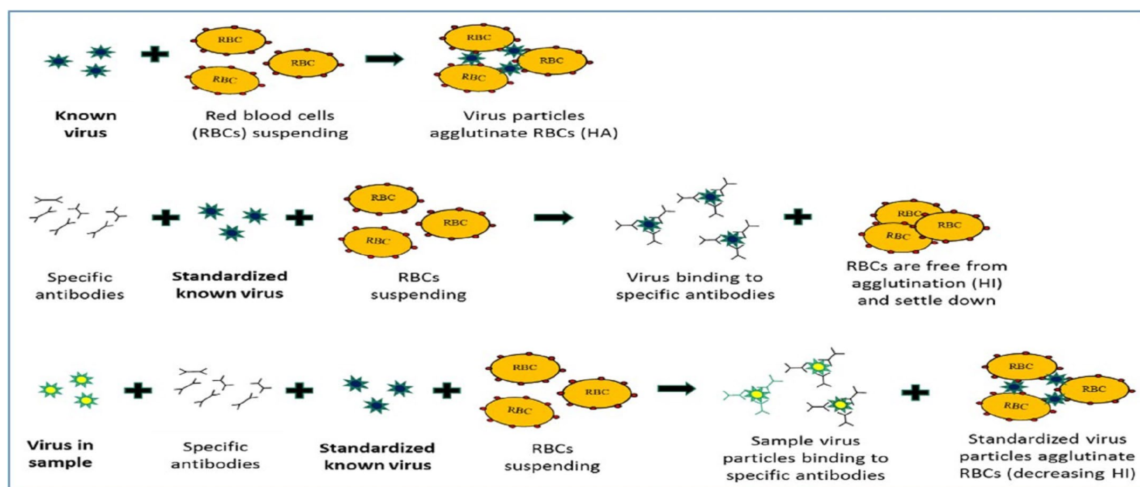

Figure 4. The principle of SSDHI. The technique is a derivative of HA (the 1st line, in respect to the mathematical signs) and HI (the 2nd line). Virus capable of binding to RBCs in HA is inhibited by specific antibodies present in the mixture of HI reaction leaving the red cells in the suspension free, which then settle to form a red point in the well's bottom. In SSDHI reactions, homogeneous virus particles in samples block specific antibodies freeing the standardized virus particles from being bound to the antibodies, so, in turn, the standard virus can agglutinate the RBCs in the suspension, and it appears first in the fourth wells of rows of 4 log_2 HI antibody serial dilution, where the concentrations of the standard serum are already diluted enough.

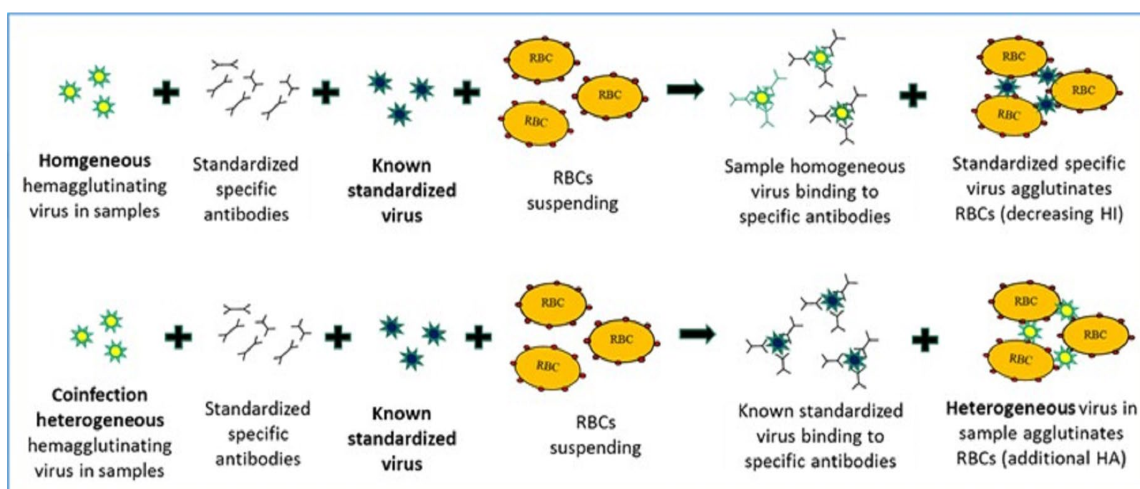

Figure 5. The schematic illustration of SSDHI phenomena of cases of the presence of specific virus (the first line) and "coinfection" with other haemagglutinating virus particles (the second line). Seemingly, there is no difference: In both cases suspending RBCs are connected via viral particles in the same mode. So, how we can distinguish the phenomena is an appealing practical problem making SSDHI much more informative.

diluted virus suspensions of the same volume cannot agglutinate the cells giving negative HA outcomes once the number of RBCs is constant. The same phenomenon could appear also in cases the virus particles are blocked by specific antibodies as in HI reactions. Conversely, the antibodies rendering the specificity of HI again may be blocked by specific virus particles present in samples, causing decline in HI titration (leftward shift) in sample rows of an SSDHI plate in comparison with the standard HI row where the same volume of saline solution was used instead of the clinical fluids. So positive SSDHI reactions do imply the presence of specific virus particles with intact haemagglutinin spikes on their surfaces and this could be supported with HA reactions. The phenomena shown in Figure 3 could serve as proof for that. In the cases of exposure to air-dryness at room temperatures, the number of particles of the virus, possibly with intact surface haemagglutinin spikes, gradually decreased as their presence could be detected with the both SSDHI and HA only in the first two days. Thus, a combination of direct HA and SSDHI reactions could help trace destabilization of every HA viruse which usually entails virus propagation in combination with a subsequent virus detection test, in which, however, exogenous viruses may overgrow tested ones and viral component molecules may remain intact for a long time after a virus has "died" and disintegrated.

The reactions are read by unaided eyes, requiring neither special microscope as in the cases of FAT/DFA (Dean and Abelseth, 1973; OIE, 2019) nor thermocycler as in the case of PCR (Mullis *et al*., 1986). The time necessary for performing the tests from pipetting saline solution into wells to the moment of completion of reading is about 40 to 60 minutes for simultaneous 11 samples, provided that all the materials for the test have been ready. For reading the reactions one should first glance at the last (rightmost) column of wells that are the indicators of the suitable moment for reading. On one plate, all the RBCs in this column of wells must settle down completely in the center of well bottom forming a red point in each well. Next, one should check the standard row of standardized 4 log_2 HI unit serum. Normally, as designed with 16 HI unit serum standard, the first four wells should have the phenomenon of inhibiting haemagglutination, that resembles the natural settlement of the cells to the well bottoms in the rightmost wells and completely differs from the three other (5th, 6th, and 7th) wells of the same row, where RBCs are connected to each other via virus particles (agglutinate) so they cannot freely settle to the well bottoms. As shown in the first picture of Figure 2, all the rows of negative samples (implying no rabies virus in the samples) appeared like the standard row. This means that while a saliva sample shows negative with SSDHI it works almost like a physiological solution with neither specific antigens nor specific antibodies in it.

Thus, the technique learnt the comparison of electrophoresis-run DNA bands (samples with positive control and/or molecular weight ladders) from the conventional PCR to formulate "shifting" and the competition of sample homogeneous virus with already known standard virus from competitive ELISA to let sample antigens compete with a constant quantity of standardized specific virus (4 HA vaccine virus), thus granting the specificity of the outcomes. It does not use any harmful substance, and

all the participatory components (saline, 1% RBC suspension, 4 HA virus, 16 HI unit rabies-specific antisera) are widely available and can be easily prepared or relatively cheap. The preparation of samples is also with quite simple procedures, almost no processing intervention is needed, so can minimize the possible losses of virus components in the samples, thus grant the sensitivity of the technique.

However, using natural clinical samples taken from animals, as a rule, must encounter complicated situations as they may contain different agents of body fluids and exudates, as well as parasites and other exogenous factors. Theoretically, we may meet phenomena of "coinfection" (term "coinfection" applied here for convenience, indeed, may be the only infection) and "contaminant antibodies", so we should foresee and distinguish the phenomena while reading SSDHI reactions. In the cases of coinfection with another HA virus, the phenomenon could be illustrated schematically as shown in Figure 5, which is seemingly not different from the presence of the specific target virus. While a sample has no specific virus, the standardized 16 HI unit specific antibodies could bind to the standardized amount of 4 HA virus particles leaving RBCs free, and in case of the presence of other kind of haemagglutinating virus(es), the red cells suspending in liquid phase could, instead, bind to those heterogeneous HA virus particles, thus joining together, and, as a rule, cannot settle to the well bottom.

So, coinfection with heterogeneous HA virus(es) could affect the technique SSDHI. However, there is a great difference between the cases of presence of specific and non-specific viruses, as we can see in the second picture of Figure 2. The appearance of additional haemagglutination reaction caused by heterogeneous virus coinfection, once present, as a rule, starts from the first (leftmost) well of any test row where the concentration of the "coinfection HA virus" is highest. And then, in higher concentration cases, the same phenomenon could appear in the next wells on the right. So we can discriminate the two phenomena of HA virus presence: specific virus should cause shifting from the right to the left, meanwhile heterogeneous HA virus(es) cause(s) shifting from the left to the right. So, with SSDHI we indeed can get additional information about possible infections of the tested animals with unidentified haemagglutinating virus, which often leads to new investigations. However, theoretically, when the coinfection virus titers are too high, that makes it impossible to discriminate phenomena of 4 $\log_2$ HA coinfection from SSDHI 4+ infection with the tested virus, we cannot perceive whether SSDHI is positive, that shows haemagglutination inhibition declination expanding leftwards from the right, or coinfection exists, that causes haemagglutination expanding rightwards from the left. So, when RBCs in all the seven wells of an SSDHI test row are agglutinated (appearance of possible SSDHI 4+ virus) it is necessary to repeat the reaction with lower (half or more diluted) concentrations of the sample fluids. However, in practice, with 1723 saliva tested for rabies for years never have we seen such a "super-positive SSDHI" phenomenon.

As seen in the fourth picture of Figure 2, the presence of additional specific antibodies in samples could also be detected in a case. The presence of specific antibodies in samples could make SSDHI detection of antigens impossible. This is considered as the main inferiority of all immunologic methods (including SSDHI, ELISA...) to template-based amplification methods, such as PCR (Mullis *et al*., 1986) and isothermal amplification techniques (Compton, 1991; Fire and Xu, 1995) in detection of pathogens. So, we should consider the type of samples for the purpose. Our studies have shown that, in all cases of no having haemorrhages, saliva fluids (and faecal extracts in water) were excellent for the purpose. The uniform appearance of all rabies negative SSDHI reactions as shown in the first picture of Figure 2 supports the statement that saliva fluids work well like physiological solutions for diluting viruses in samples. And, the outcomes of both SSDHI and HA with virus extracted from filter paper discs in the first and second pictures of Figure 3 showed that a combination of the two techniques could help detecting the sustainable, or intact, virions in environments. This destabilization research of rabies virus showed that air-dried virions of the pathogen can sustain intact at room temperatures for about 2 days.

A titration in practice is determined through serial dilutions, and it demonstrates only the lowest values representing an entire categorical range of continuous values, as 4 $\log_2$ HI serum titre, for example, represents values from 16 HI units up to nearly 32 HI units. With so high discrepancies of real values of titres, different batches of standard sera could be "neutralized" with different numbers of specific virus particles or antigen equivalents, that are enough to make a change from one category of logarithmic scale titre to another one in the test outcomes. So, without necessary standardization, SSDHI tests with different 4 $\log_2$ HI sera would naturally give different levels of sensitivity. So, we should adjust the concentration of antibodies as it was done exemplarily here with "saline ten-percent replacement series" shown in Table 1. By the way, because of the different scales between logarithmic categories, it is better to designate positive results with "plus(es)" as (SSDHI 1+, 2+...). Theoretically, SSDHI 1+ results would span over a range from above 0 to nearly 8 titre units (enough for conversing a quantity of above 16 HI units of antibodies to under 16 HI units but above 8 HI units), while SSDHI 2+ results from above 8 to 12, and SSDHI 3+ from above 12 to 14 titre units of virus. So, the reaction could not be used to indicate the intensities of infections or virus-shedding. Therefore, for determining these parameters, simple HA tests using samples with same 1% RBC suspension should be additionally implemented.

Visualization of the HA reactions is the most important criterion of the tests, and it depends much on the number of the colorful cells. The proportions between reagents (RBCs with virus particles) decide the appearances of reactions. RBCs cannot agglutinate while connected with too small number of virus particles because of disproportionateness of their quantities. In turn, decreasing the number of the cells to proportionate with the number of virus particles could restore the phenomenon of agglutination. The more the number of RBCs is, the more the standard virus particles could be bound with in HA, and, so in HI, the more the number of specific antibodies could be in need to "inactivate" the virus, consequently in SSDHI, the more the number of virus particles in the samples could be fit to "compete" for the standardized antibodies and thus exhibit implicitly their own presence through freeing the standardized virus' activities with RBCs. Conversely speaking, minimization of the number of RBCs in every well of reaction plates could increase the sensitivity of SSDHI. However, decreasing the number of RBCs, through decreasing either their concentrations or reacting volumes, harms capability of reading reactions since the sediment of RBCs in negative HA cases (in the rightmost wells) could be too small to visualize, or in the cases of quite low concentrations of the RBCs, because of far distances between them in suspensions they could not even form tridimensional lattices large enough for observation while being agglutinated. So, absolute number of RBCs does the primary inner factor decisively determine the others, and their reasonable concentrations and volumes confer the sensitivity, readability, and comparability of the HA-HI-SSDHI system. For that, we should maintain the constancy of both the concentrations of RBC suspension and volumes of them in each well of reactions to grant the comparability of outcomes of the reactions. Empirically, 25 µL of defined 1% RBC suspension for each well of reaction in series of HA, HI, and SSDHI is a well-working quantity suitable for readability of tests with the same volumes of all the other factors (virus suspensions, sera, and samples). Besides, for maximizing the sensitivity of SSDHI we should also adjust the quantities of specific antibodies to get standard 16 HI unit sera.

In regard to the rabies infection investigation, the prevalence of the virus revealed in Quang Binh and Thua Thien Hue was about 3% with no clear difference (p=0.845) between the areas, and this almost coincided with the fact that "in the last decade, Vietnam reported a total of 914 human deaths, averaging about 91 deaths each year" (CDC, 2018). So, for the elimination of the disease, many efforts should still be required, including

heightening capacity of veterinarians for implementing prophylaxis and recognizing the threats through testing the presence of the pathogen. For that, SSDHI is a suitable alternative method of rabies detection in animal saliva fluids.

CONCLUSION

Haemagglutination property of viruses was proven more important as with the case of rabies virus shown here, we could utilize it for determining virus titres in vaccines and virus-specific antibody titres in sera, as well as for checking the presence of the pathogen in clinical samples with a broadened application technique of Assay of Shifting of Standardized Direct Haemagglutination Inhibition (SSDHI). Besides being able to detect rabies virus in saliva fluids for diagnosis and epidemiological investigation of the infection, SSDHI could provide additional information on heterogeneous haemagglutinating virus "coinfection" and antibody "contamination" of the tested animals and be useful in checking destabilization of the HA virus. With it, good coincidence of test results with clinical cases of furious rabidity of dogs was proven, and our epidemiological investigation from 2017 to 2021 showed that the rabies infection rate in Central Vietnam was about 2.84%. Meanwhile, combination of SSDHI and HA showed that air-dried virions of rabies virus could sustain intact at room temperatures about 2 days. Regarding to technical aspects, as a decisive primary factor of sensitivity, readability, and comparability of the techniques of HA, HI, and SSDHI, a permanent volume of 25 μL of 1% RBC suspension for each well of the reactions should be applied.

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CONFLICT OF INTEREST

There are no conflicts of interest to declare.

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