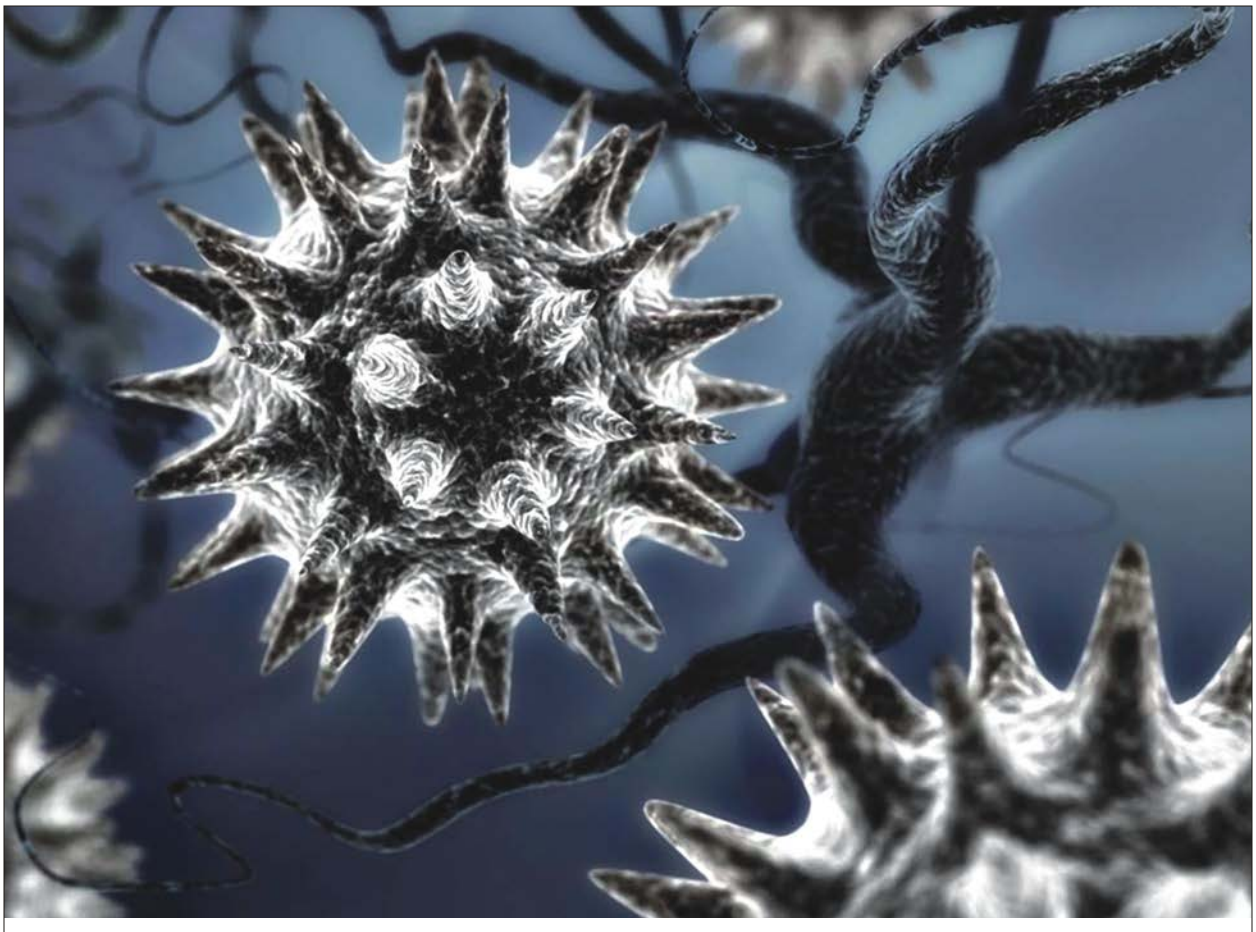

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Field Diagnosis of Avian Influenza H5N1 Virus in Egypt using RAPID Immunochromatographic Diagnostic Tests

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ABSTRACT

Rapid on-spot testing for influenza virus can greatly assist in the clinical management decision. In Egypt, where avian influenza A virus H5N1 is now endemic and human infections are on the rise, rapid H5N1 detection in infected poultry is essential for controlling spread to human. To test the performance of two rapid influenza tests for the detection in naturally infected birds, clinical specimens from different species and organs of sick and dead birds collected during 2007 and 2009 in Egypt were tested using virus culture in embryonating chicken eggs and RT-PCR as references. Influenza rapid tests efficiently detected H5N1 in clinical samples derived from different organs including lung, liver and brain tissues with high sensitivity and specificity. The sensitivity and specificity were 71 and 100% for Sysmex avian influenza kit and 86 and 33% for Sysmex influenza A/B kit. Thus, Sysmex avian influenza kit was shown to be a potentially useful tool for the direct and rapid detection of H5N1 in clinical specimens due to its high specificity. Additionally, an advantage over the other kit is specifically reacting with viruses of avian origin but not human or even swine origin, thus proving worthy for discriminating influenza A infections.

Key words: Influenza, rapid detection, HPAI H5N1, Egypt, avian, sysmex

INTRODUCTION

Highly pathogenic avian influenza (HPAI) H5N1 virus in Egypt was first detected in February 2006 causing massive disease outbreaks in poultry populations in many geographical regions (WHO, 2009). In 2009, human infections were not linked to poultry outbreaks; they were individually reported with high incidence in toddlers (NAMRU-3, 2009). Human infections in Egypt are all linked to a history of direct contact with a sick or a dead bird and in all cases the birds' species was never identified. As the virus infections in human are on the rise, many cases are presented to hospitals suspecting a possible H5N1 infection. Thus, discrimination of influenza infections in human is essential in order to economically and wisely apply subsequent treatments and avoid unnecessary interventions.

Field monitoring of influenza virus infections can be rapidly achieved by testing the presence of influenza antigens using anti-nucleoprotein (NP) antibodies. For that, rapid diagnostic tests for influenza viruses are widely available; detecting and distinguishing influenza A and B viruses or only detecting influenza A viruses or influenza viruses in general; A and B without discrimination (Ghebremedhin *et al*., 2009). The tests differ further in the time required for yielding a result, the type of specimens appropriate for the test and the cost. Sysmex influenza A/B and Sysmex avian

influenza utilize NP-specific monoclonal antibodies against human influenza A and B and against avian influenza viruses, respectively. However, with the high incidence of avian influenza infections in human and the pandemic threats of avian origin, rapid and specific identification of avian influenza infection in poultry, mainly H5N1 is a priority. Further, with the high spread rate of the HPAI H5N1 virus infections in Egypt, surveillance and monitoring the distribution and activity of influenza viruses in general is intensively needed.

The aim of our study was to evaluate Sysmex influenza A/B (Sysmex-H) and Sysmex avian influenza (Sysmex-A) kits for the sensitivity and specificity for differential detection of avian influenza H5N1 antigens from naturally infected birds in respiratory and non-respiratory samples in Egypt without previous *in vivo* or *in vitro* amplification.

MATERIALS AND METHODS

Clinical specimens, viruses and viral culture: Ten samples including respiratory organs (lungs and tracheae) and non-respiratory organs (brain and liver) were collected from chicken and ducks naturally infected with HPAI H5N1 in Egypt between 2007 and 2008. The samples were homogenized separately to a 10% final concentration in phosphate-buffered saline containing antibiotics cocktail and stored at -80°C till use. Field samples were collected from different bird species; chicken, turkey and pigeons then either transferred in transport medium to the laboratory or tested *in situ*.

For virus isolation, 11 day old specific pathogen-free Embryonated Chicken Eggs (ECE) were inoculated with the same tested aliquots.

Human influenza A viruses; A/Beijing/262/95 (H1N1) and A/Panama/2007/97 (H3N2), avian influenza A viruses; A/Duck/Ukraine/1/63 (H3N8) and A/Duck/ HK/342/78 (H5N2) and swine influenza A virus; A/swine/ Hokkaido/2/81 (H1N1) were used.

Influenza rapid tests: Influenza rapid tests (Sysmex-H and Sysmex-A) from Sysmex Corporation, Hyogo, Japan were used. The samples were mixed with the detergent solution at 2 and 5% final concentrations. Filtered detergent-sample solution was applied to each kit according to the manufacturers' protocols. The reactivity was determined by visual color development.

RNA purification and RT-PCR: Total RNA was extracted from the tissue homogenates using TRIZOL reagent (Invitrogen, Japan) according to manufacturer's instructions. RT-PCR amplification of the H5N1 nucleoprotein (NP) was carried out as previously (Lee *et al*., 2001) using our specific primers.

Determination of focus-forming units (ffu): Madin-Darby canine kidney (MDCK) cell monolayer in 96-well plate was infected in quadruplicates and performed for the determination of ffu as previously (Okuno *et al*., 1990).

Statistical analysis: Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for each kit with tissue culture or RT-PCR as the standard using two-by-two contingency tables.

RESULTS

Comparison of the Sysmex-A test to ECE culture: Seven out of 10 samples (70%) were antigen-positive for avian influenza A virus by ECE culture (Table 1). Of these, 5 samples (50%)

were positive by the Sysmex-A test at 5% (Fig. 1a) and 2% (data not shown) sample dilutions. The Sysmex-A positive samples were ECE positive as well as RT-PCR positive for the nucleoprotein (NP); however, the 10 samples were positive for the NP by RT-PCR. Further, negative results were

Table 1: Summary of laboratory tested samples

Samples	ECE[a]	Sysmex-A	Sysmex-H
Chicken1 lung	Pos	Pos	Pos
Chicken1 liver	Pos	Pos	Pos
Chicken1 trachea	Pos	Pos	Pos
Duck1 brain	Pos	Pos	Pos
Duck1 liver	Neg	Neg	Pos[b]
Duck1 trachea	Neg	Neg	Neg
Duck2 brain	Pos	Neg	Pos
Duck2 liver	Pos	Neg	Neg
Duck3 lung	Pos	Pos	Pos
Duck3 liver	Neg	Neg	Pos[b]
H1N1 (human)	Pos	Neg	Pos
H3N2 (human)	Pos	Neg	Pos
H5N2 (duck)	Pos	Pos	Pos
H1N1 (swine)	Pos	Neg	Pos

ECE: Embryonating chicken egg; Pos: Positive; Neg, negative; ND, Not done, [a]Isolation in 11 day-old ECE, [b]Weak band intensity

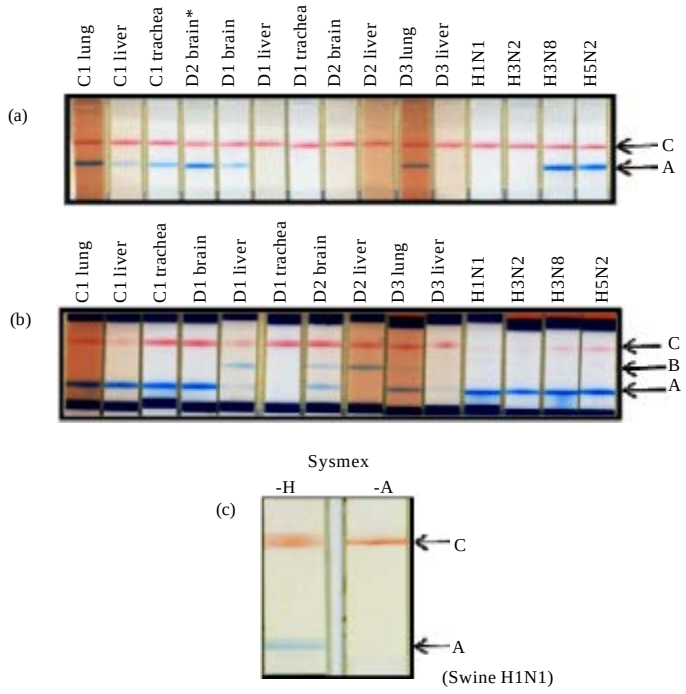

Fig. 1: Detection of influenza A antigens by Sysmex-A and Sysmex-H. Same samples were applied in a 5% final concentration to (a) Sysmex-A and (b) Sysmex-H kits according to the manufacturer's instructions. (c) Detection of A/swine/Hokkaido/2/81 (H1N1) by both kits. C: Control line, a: Influenza a, b: Influenza b. c1: Chicken 1, d1,2,3; duck 1,2,3. *Allantoic fluid-derived sample from D2 brain. H1N1; A/Beijing/262/95, H3N2; A/Panama/2007/97, H3N8; A/Duck/Ukraine/1/63, H5N2; A/Duck/HK/342/78

Table 2: Performance of the rapid diagnostic kits compared to ECE culture

		ECE						
Kit		Pos	Neg	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	RT- PCR[a]
Sysmex-A	Pos	5	0					
	Neg	2	3	71	100	100	60	10/10
Sysmex-H	Pos	6	2					
	Neg	1	1	86	33	75	50	10/10

Pos: Positive; Neg: Negative; PPV: Positive predictive value; NPV: Negative predictive value, [a]The ten samples were all positive by RT-PCR for the NP gene segments

Table 3: Field performance of the Sysmex-A kit as compared to RT- PCR

		RT-PCR						
Kits result[a]	No. of samples	Pos	Pos	Sensitivity (%)	Specifi city (%)	PPV (%)	NPV (%)	p-value
Pos	13	12	1	92	98	92	98	<0.0001
Neg	46	1	45					

PPV: Positive predictive value; NPV: Negative predictive value.[a]Sysmex-A rapid diagnostic kit

detected with duck-derived samples only. The sensitivity of the Sysmex-A compared to ECE culture was 71% with a specificity of 100%. The PPV was 100% and the NPV was 60% (Table 2). Importantly, the Sysmex-A reacted with influenza viruses of avian origin; H3N8 and H5N2 but did not show any reaction with human influenza viruses, H1N1 or H3N2 (Fig. 1a) or swine H1N1 virus (Fig. 1c).

Comparison of the Sysmex-H test to ECE culture: Out of 10 samples, 8 (80%) were positive for influenza A virus in the Sysmex-H test while 7 (70%) were positive by ECE culture (Table 1). The sensitivity, specificity, PPV and NPV of the Sysmex-H were 86%, 33%, 75% and 50%, respectively (Table 2). Although Sysmex-H showed higher sensitivity than Sysmex-A, it showed non-specific reaction with influenza B-positive line in 4 samples; duck1 liver, duck2 brain duck2 liver and duck3 lung (Fig. 1b). In contrast, Sysmex-A showed 100% specificity. Further, two samples were positive by Sysmex-H and negative by ECE isolation but were positive by RT- PCR. In addition, as Sysmex-A, the negative detections were with the duck samples only. Such negative results detected by both kits could be related to virus titers under the detection limit in the clinical materials especially in duck samples, while the chicken samples had >0 ffu/mL (data not shown).

Field performance of the sysmex-A: Tissue samples from chicken (n = 20) and turkey (n = 4) and throat swabs from pigeons (n = 35) were collected from different localities in Egypt then tested by Sysmex-A and RT-PCR in parallel. The sensitivity, specificity, PPV and NPV were 92, 98, 92 and 98%, respectively (Table 3). The pigeon samples were all negative by Sysmex-A and RT-PCR as well, while the chicken and turkey had 60% and 25% kit-positive rates, respectively.

DISCUSSION

High sensitivity and specificity of a rapid diagnostic kit is essential for accurate diagnosis of influenza virus infection. However, discrimination of influenza subtype is of high importance in the era of possible pandemic threat of HPAI virus H5N1 followed by the newly emerged H1N1 swine influenza. In our study, the Sysmex-A rapid diagnostic kit offers a potentially useful tool for

direct detection of avian influenza antigen in clinical specimens. Although nasopharyngeal swabs from human are recommended, the field sensitivity and specificity with our specimens were high (92 and 98%, respectively). The kit was further proven to be avian influenza specific where negative reactions were detected with influenza viruses of human, H1 and H3 as well as swine origin. Although the Sysmex-H had 86% sensitivity, non-specific reactions could be detected in the influenza B positive test line and were mainly with samples from ducks. However, the reason for such non-specific reactions could be that Sysmex-H is optimized for use with nasopharyngeal swabs from human only. Further, all the samples were used without prior clarification of cellular debris or blood and this did not seem to affect the assay specificity for Sysmex-A and Sysmex-H, where clear reaction could be detected in the influenza A positive test lines indicating a feasibility of sample usage.

Although, the standard for detecting influenza virus infection is viral isolation (Booth *et al.*,2006) and RT-PCR is widely used as well, they require more time to produce a confirmative result, high skills and complex infrastructures delaying the application of post-diagnostic decisions. In contrast, rapid influenza diagnostic tests offer quick results and no skills or specific infrastructures are required. The development of the results did not take 5 min for the two kits used in our study, even that it is noted for each 10 to 20 min from sample application to the development of a positive reaction line. Further, testing procedures were easily performed without any specific requirements and at low cost compared to gold standard analysis.

Sysmex-H has been shown to have type-specific high sensitivity and specificity for the detection of influenza A (Hamamoto and Asai, 2007) and B viruses (Hara *et al.*, 2005; Takahashi *et al.*, 2005; Hamamoto and Asai, 2007), however, it does not discriminate between the influenza A virus subtypes. Even though Sysmex-A cannot discriminate between avian influenza viruses, it can with high specificity identify influenza viruses of avian origin making it a kit of choice for rapid field diagnosis. Despite the high specificity and lack of false-positive results for Sysmex-A, in an endemic environment like that of H5N1 in Egypt negative results should be further confirmed by culture or RT-PCR. In contrast, because of a high PPV (100%), positive Sysmex-A results do not necessarily require additional confirmation other than virus typing.

Application of influenza rapid diagnostic tests for avian influenza viruses has been evaluated previously (Bai *et al.*, 2005; Fedorko and Nelson, 2006; Zarkov, 2008a, b; Ghebremedhin *et al.*, 2009). Although, Directigen FLU-A was effective for the detection of animal influenza viruses from ducks and swine (Ryan-Poirier *et al.*, 1992), they were sensitive with no subtype discrimination. Others showed lack of satisfactory results with an avian influenza virus using the same kit (Woolcock and Cardona, 2005). Our results differ in using a kit that specifically identified avian influenza A virus; H5N1 in naturally infected birds.

The rapid diagnosis of influenza viruses in hospital settings where avian influenza infection is suspected is important for infection control, application of potential therapy and avoiding costly interventions as well. Further, specific detection of avian and not human or swine influenza A viruses by Sysmex-A indicates that it can be a worthy diagnostic aid if applied to human point of care units, especially with the recent spread of newly emerged swine-origin pandemic influenza virus H1N1. Moreover, this kit can be consistently applied in the field for the surveillance of avian influenza viruses in bird and animal hosts that could represent a human threat.

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