系統抗体遺伝子群に特異的な各種プライマー配列の選定と、解析プログラムのレファレンス配列の改変を行った。

3）抗体L鎖の解析アルゴリズムの策定：昨年度は、抗体H鎖の解析アルゴリズムを策定したので、本年度はそれを抗体L鎖の解析に拡張した。抗体L鎖の各遺伝子配列の同定・整理に多くの労力を費やした。

4）記憶B細胞の長期保持に関与する分子の解析：これまでの研究で、ワクチンが誘導する免疫記憶B細胞の長期間保持に関与する分子として、カドヘリン17分子を同定していた。本年度は、カドヘリン17遺伝子ノックアウトマウスの解析をさらに進めた。

5）抗体遺伝子合成による抗体分子の発現系の確立：上述の、NGSを用いた抗体レパートリーの網羅的解析法方法で同定した、目的とする抗体重鎖遺伝子（複数）および抗体軽鎖遺伝子（複数）について、抗体可変領域（Vドメイン）のDNA配列を遺伝子合成する。この合成DNAを抗体定常領域（ヒト型）を有する発現ベクターに組み込み、培養哺乳類細胞（CHO細胞等）に遺伝子導入して抗体タンパク質を発現し、培養上清中に分泌された抗体分子を精製してその性状を解析する。この方法により、NGS解析アルゴリズムで突き止めたワクチン抗原特異的抗体遺伝子の抗原（エピトープ）反応性をウェットな実験で確認する解析法を確立する。

6）製造方法の異なるワクチンによる異なる抗体応答の網羅的解析：ワクチン製造のために発育鶏卵での増殖性を確保するウイルス継代馴化を行った結果生ずる宿主馴化変異によるワクチン有効性の低下の原因を知るために、発育鶏卵ワクチンをBALB/cマウスに免疫して応答する抗体レパートリーと、培養細胞法ワクチンを免疫して応答する抗体レパートリーを、NGS解析アルゴリズムで比較した。

C．研究結果

1）抗原特異的抗体レパートリーの抗原親和性成熟に伴う系統樹解析：NP-CGGを5匹のC57BL/6マウスに免疫して、個体ごとにNGS解析アルゴリズムで比較した。その結果、NPエピトープ特異的に応答する抗体群を網羅的に同定することが出来、それらのうち、最もメジャーな抗体遺伝子がVh186.2である事が明らかになった。Vh186.2遺伝子は、ハイブリドーマ法を使った多数の論文報告から、NPエピトープに反応する主要な抗体遺伝子である事が知られている。このことは、本研究で開発した、NGS解析アルゴリズムが特異抗原に応答する抗体遺伝子を感度よく発見した事を示している。Vh186.2遺伝子は、マウスの脾臓に形成される胚中心の中で体細胞突然変異を蓄積して、抗原親和性成熟を起こす。この過程を詳細に検討するため、NGSで得られた約1,500リードのVh186.2遺伝子配列を解析した。産総研の加藤和貴等が開発したMafftプログラムでマルチプルアラインメントを行った後、産総研の藤博幸が開発したDrawTreeプログラムで系統樹解析を行った。その結果、NPエピトープとの結合性を強化する突然変異であるW33LとHY99Gの発生ダイナミクスを可視化することが出来た。この事は、ワクチンを免疫した後、感染防御に働く抗体群がどのように成熟して抗体力価と抗原エピトープ中和能の上昇を引き起こすかの解析が、本方法で可能である事を示している。

2）BALB/c系統マウスの抗体遺伝子座の解析と抗体レパートリーの網羅的解析：インフルエンザワクチンの力価試験に用いられるマウス系統はBALB/cであり、C57BL/6系統のマウスとは異なる抗体遺伝子座の構成を持つ。C57BL/6系統マウスと異なり、BALB/cマウスの抗体遺伝子座の詳細な解析は未だになされていない。そこで、BALB/cマウスの脾臓RNA全体に対してNGS解析を行い、データバンクにあるBALB/c抗体遺伝子配列と対応させた。上位110個の抗体IgH鎖遺伝子を選定する事により、脾臓RNA中の抗体配列を十分カバーすることが出来た。この110個のBALB/c抗体遺伝子群に特異的な各種プライマー配列の選定を行い、それに伴う解析プログラムのレファレンス配列の改変を行った。

3）抗体L鎖の解析アルゴリズムの策定：マウスの抗体L鎖はカッパ鎖(Ig-κ)とラムダ鎖(Ig-λ)から成り、それぞれ1：19の比率で存在する事が知られている。カッパ鎖について、Vκ遺伝子を101個、Jκ遺伝子を4個、ラムダ鎖について、Vλ遺伝子を3個、Jλ遺伝子を3個、選定する事により、ほぼ全ての抗体L鎖配列を分類することが出来た（抗体L鎖遺伝子のアノテーション）。これらの遺伝子群の遺伝子再構成による組み合わせが起こるが、それに対応した個々のL鎖配列のリード数をヒストグラムで可視化するプログラムを作成した。これにより、マウス個体内の全ての抗体L鎖を網羅的に解析する準備が整った。個々の抗体L鎖がどの抗体H鎖とペアを組んでいるかについては、興味ある問題であり、それを解決するアルゴリズムの考案を試みている。

4）記憶B細胞の長期保持に関与する分子の解析：カドヘリン17分子は、抗原免疫に誘導される免疫記憶B細胞の長期間保持に関与する。本研究で、カドヘリン17遺伝子ノックアウトマウスでは、再度の抗原刺激による2次抗体応答が顕著に抑制され、それがカドヘリン17分子陽性記憶B細胞の数の低下に因る事を示した。さらに、カドヘリン17分子が、記憶B細胞の長期生存を可能にする免疫組織内微小環境を構成する、重要な因子である事を明らかにした。このことは、ワクチン接種による、免疫記憶の植え付け分子機序の一端を明らかにした事を意味し、これを応用すれば、ワクチン効果の増進が期待できる。

5）抗体遺伝子合成による抗体分子の発現系の確立：上述の、NGSを用いた抗体レパートリーの網羅的解析法方法で発見した重要な抗体H鎖遺伝子および抗体L鎖遺伝子について、NGSの配列情報から抗体遺伝子を合成する事により、実際に抗体タンパク質を作製することができた。この方法では、抗体の定常領域ドメインはヒトのIgG1のものを用いている事から、抗体医薬への応用も可能となる。この方法を用いて、これまで知られていなかった、NP-CGG抗原のキャリアー部分（CGG, Chicken gamma globulin)に対する抗体遺伝子がVh9-1であり、実際にVh9-1遺伝子合成によって作製した抗体がCGGに結合する事を明らかにした。このことは、ワクチン接種によって誘導される抗ウイルス抗体を本研究で開発したNGS解析アルゴリズムで調べれば、ワクチンの免疫原性を発揮する全ての抗体群を網羅的に同定し評価し、さらにそれらを遺伝子合成法で作製して、例えば抗体医薬として用いる事が可能となった事を示す。

6）製造方法の異なるワクチンによる異なる抗体応答の網羅的解析：発育鶏卵ワクチンと培養細胞法ワクチンをBALB/cマウスに免疫して、応答する抗体レパートリーをNGS解析アルゴリズムで比較した。その結果、IgG1クラスとIgG2aクラスの抗体について、育鶏卵ワクチンと培養細胞法ワクチンで共通に誘導される抗体レパートリー、および、それぞれに特異的に誘導されて来る抗体レパートリーについて網羅的に可視化することが出来た。この解析過程で、IgG1クラスよりはIgG2aクラスの抗体群の方が、インフルエンザワクチンに対する応答が強い事が明らかになった。このことは、最近の論文報告で明らかになった知見である、IgG2a抗体のウイルス抗原指向性の事実と一致する。すなわち、本研究で開発したNGS解析アルゴリズムによって、異なる製造法によるワクチンが異なる免疫原性を有する事を、抗体分子レベルのダイナミクスで明示する事により、初めて詳細に明らかにした。

D．考察

ワクチンの免疫原性が直接に反映される抗体レパートリーの応答ダイナミクスを網羅的に解析する新規手法を確立し、その深化と応用を行った。

抗原特異的抗体レパートリーの抗原親和性成熟の過程を正確に解析し可視化する方法を検討した。免疫学で多用されるNP-CGG抗原をC57BL/6マウスに免疫して、個体ごとにNGS解析アルゴリズムで比較した。主要な応答抗体遺伝子がVh186.2である事を確認した。このことは、我々独自のNGS解析アルゴリズムが特異抗原に応答する抗体遺伝子を感度よく発見した事を示している。この過程で、抗体応答遺伝子の発見にはNGSのリード数が5,000リード程度で十分である事を確認した。また、抗体の抗原親和性成熟の発生ダイナミクスを可視化するにも成功した。ワクチンの免疫原性を抗体レパートリーの親和性成熟のダイナミクスとして評価する事が、本方法で可能である事を示された。

抗体遺伝子座の解析と抗体レパートリーの網羅的解析を行う事により、本解析法を、インフルエンザワクチンの力価試験に用いられるマウス系統であるBALB/cにも拡張した。現在、解析対象をマウスからヒトに拡張する準備を進めている。

抗体L鎖の解析アルゴリズムを確立した。これにより、マウス個体内の全ての抗体L鎖を網羅的に解析する事が出来る。これを応用して、個々の抗体L鎖がどの抗体H鎖とペアを組んでいるかを決定できるアルゴリズムの創出を試みている。これに関連して、抗体H鎖との結合性が、H鎖の個性に影響されないような、いわゆるユニバーサルL鎖遺伝子の作製も試みる必要がある。

記憶B細胞の長期保持に関与する分子として、カドヘリン17分子の作用機作を明らかにした。今後、これをどのように応用すれば、ワクチン効果の増進が期待できるか検討する必要がある。

抗体遺伝子合成による抗体分子の発現系を確立した。ワクチン接種によって誘導される抗ウイルス抗体レパートリーのうち、有用性が高いと期待できる抗体遺伝子を遺伝子合成法で作製して、例えば抗体医薬として用いる事が可能となった。このことは、例えば、インフルエンザ

ウイルス亜群に共通なエピトープを認識する抗体を同定し、それを抗体医薬として用いる事にも応用できる。

発育鶏卵ワクチンと培養細胞法ワクチンの応答抗体レパートリーを本 NGS 解析アルゴリズムで網羅的に比較する事により、ワクチンの異なる免疫原性を、抗体分子レベルの詳細なダイナミクスで明示することができた。この、発育鶏卵ワクチンと培養細胞法ワクチンの免疫原性の差異を最終的に明らかにするために、主要な応答抗体遺伝子の合成をすでに終えており、それらの反応性を今後詳細に検討して行く。

E．結論

ワクチンの免疫原性を詳細にまた定量的に解析するために、次世代シークエンサを用いて抗原特異的な抗体群を網羅的に知る新しい方法を確立した。この新規方法で、ワクチン免疫により誘導される全ての抗体レパートリーとその存在量を網羅的かつ定量的に解析する事が出来る。また、ワクチン接種によって誘導される抗ウイルス抗体レパートリーのうち、有用性が高いと期待できる抗体遺伝子を遺伝子合成法で作製して、例えば抗体医薬として用いる事が可能となった。本方法を用いて、ワクチンやワクチン製造株の特性に応じた性能を評価する品質管理試験を確立すれば、ワクチンが一定の品質で供給され国民の健康や医療費の抑制などに貢献できると考える。

F．研究発表

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G．知的財産権の出願・登録状況

1．特許取得
なし

2．実用新案登録
なし

3.その他
特になし。

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Development of a sensitive novel diagnostic kit for the highly pathogenic avian influenza A (H5N1) virus

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Abstract

Background: Sporadic emergence of the highly pathogenic avian influenza (HPAI) H5N1 virus infection in humans is a serious concern because of the potential for a pandemic. Conventional or quantitative RT-PCR is the standard laboratory test to detect viral influenza infections. However, this technology requires well-equipped laboratories and highly trained personnel. A rapid, sensitive, and specific alternative screening method is needed.

Methods: By a luminescence-linked enzyme immunoassay, we have developed a H5N1 HPAI virus detection kit using anti-H5 hemagglutinin monoclonal antibodies in combination with the detection of a universal NP antigen of the type A influenza virus. The process takes 15 minutes by use of the fully automated luminescence analyzer, POCube.

Resutls: We tested this H5/A kit using 19 clinical specimens from 13 patients stored in Vietnam who were infected with clade 1.1 or clade 2.3.4 H5N1 HPAI virus. Approximately 80% of clinical specimens were H5-positive using the POCube system, whereas only 10% of the H5-positive samples were detected as influenza A-positive by an immunochromatography-based rapid diagnostic kit.

Conclusions: This novel H5/A kit using POCube is served as a rapid and sensitive screening test for H5N1 HPAI virus infection in humans.

Keywords: H5 hemagglutinin, Highly pathogenic avian influenza virus, Rapid influenza diagnosis, Monoclonal antibody, Clinical specimens

Background

Influenza viruses belong to the *Orthomyxovirus* family, whose genome is composed of eight segments of negative-sense RNA encoding 12 proteins. Two major glycoproteins, hemagglutinin (HA) and neuraminidase (NA), are located on the viral envelope, and 16 HA subtypes and 9 NA subtypes of avian influenza A have been identified on the basis of their antigenicities [1,2]. There are three types of influenza virus, namely, A, B, and C. Influenza A (both H1N1 and H3N2 subtypes) and influenza B viruses circulate among the human population each year and are the causative agents of seasonal flu. There have been several pandemics of influenza A infections, which have resulted in the deaths of many humans and animals [2,3]. The high variability of influenza A viruses is driven by frequent mutations in genomic RNA (drift) and by genetic reassortment among avian, porcine, and human strains [4]. This has hampered the development of a universal cross-protective flu vaccine.

The 2003 and 2004 outbreaks of the highly pathogenic avian influenza (HPAI) virus of subtype H5N1 that occurred in poultry and wild birds were genetically traced back to the H5N1 HPAI virus that caused the first outbreak in Hong Kong in 1997, and also those of 2001

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and 2002 [5]. This report stated that although the H5N1 HPAI virus remained endemic to that region, it had the potential to become pandemic. The H5N1 HPAI virus has occasionally crossed the species barrier to humans in Asia, resulting in human fatalities [6,7], the first of which was recorded in Vietnam in December 2003 [8]. Numerous clinical cases of H5N1 HPAI virus infections have since been reported in Vietnam, and the disease has spread to other countries in Southeast Asia and the Middle East such as Indonesia, Cambodia, Thailand, Egypt, and Turkey [7,9].

The H5N1 HPAI virus has evolved into many phylogenetically distinct clades and subclades, and these diverse lineages have been largely geographically separated since 2005 [9]. During 2007 in northern Vietnam, the clade 1 virus was displaced by the clade 2.3.4 strain that has a different antiviral susceptibility profile [10,11]. The diversity of this virus is continuing to expand [3,12,13]. Although the earlier endemic outbreak of the avian H5N1 HPAI virus appears to be under control, the threat of a human influenza pandemic remains.

PCR-based molecular tests are one of the most sensitive ways to detect the influenza virus, and conventional and real-time RT-PCR methods have been developed to diagnose H5N1 HPAI virus infections in humans [14-16]. However, only centralized and well-equipped laboratories with trained personnel can perform these analyses. Viral antigen detection using antibodies (Abs) offers an easier and quicker diagnostic test; however, commercially available rapid detection kits for influenza A and B have poor clinical sensitivity for the identification of H5N1 HPAI infection [9]. Using previously prepared monoclonal antibodies (mAbs) against influenza A virus HA of the H5 subtype [17], we developed a rapid, sensitive, and H5N1 HPAI virus-specific diagnostic test kit for H5 HA in combination with detection of the universal NP antigen of type A influenza (H5/A kit), which is processed by a compact and fully automated luminescence analyzer, POCube (Toyobo Co Ltd., Osaka, Japan). In this study, we evaluated this novel H5/A diagnostic kit using clinical specimens infected with the H5N1 HPAI virus (genetically confirmed) in Vietnam and demonstrated the sensitive dual detection of H5 HA and type A nucleoprotein (NP) antigens for the first time. Despite of a limited number of available H5N1 clinical specimens, our results strongly suggest that this diagnostic test is a useful tool in the rapid and reliable identification of H5N1 HPAI virus infections.

Methods

POCube system

The POCube is a fully automated and compact immunological analyzer developed by Toyobo Co. Ltd, which is intended to support the Point-of-Care Testing (POCT) system in clinics. The POCube analyzer is relatively small (280 × 310 × 275 mm), easy to operate, and rapidly measures antigen-Ab complexes by the sensitive detection of luminescence (Figure 1). The POCube system has been used in clinics to detect C-reactive protein (CRP), prostate antigen, influenza A and B viruses, respiratory syncytial virus, in combination with kits authorized by the Ministry of Health, Labour and Welfare in Japan. The principle of the POCube system is to use two mAbs and/or polyclonal Abs that have distinct specificities, where one is biotinylated and the other is conjugated to alkaline phosphatase (ALP). The immune complexes are trapped by an anti-biotin Ab-coated filter membrane in a reaction vessel and the activity of ALP is measured by luminescence output. The whole process takes 5–15 min (depending on the kit), the result is expressed as a luminescence count, and a clinical diagnosis is indicated as "positive" or "negative".

The H5/A kit consists of one cartridge containing two reaction vessels with an anti-biotin Ab-coated filter membrane and following solutions in each compartment of the reservoir tank sealed with an aluminum sheet (Figure 1); a biotinylated anti-H5 HA mAb and a ALP-conjugated anti-H5 HA mAb solutions to detect H5 HA antigens, a commercially available biotinylated and ALP-conjugated mAb set against the type A influenza NP antigen, washing buffer, and a luminescent substrate (APS-5, Lumigen, Inc., Southfield, MI). Up to 180 μl of sample is placed into the first empty reservoir tank within the cartridge. When the POCube operation is started, the sample solution is transferred to reservoir tanks containing each set of Abs and allowed to react at 40°C before the immune complex consisting of antigen, biotinylated Ab and ALP-conjugated Ab is formed. Each reaction solution is then transferred to each vessel and trapped on an anti-biotin Ab-coated filter membrane. The filter membrane is washed with buffer in the tank, substrate is added to each vessel, and the chemiluminescence is measured. The program is set to indicate a positive or negative result for H5 HA and type A influenza virus antigens based on a predetermined cut-off index set that is calculated by adding four standard deviations (SDs) to the average of eight measurements for the negative control. The luminescence counts for eight negative controls in the lot used to test clinical specimens from Vietnam were 1939–2717 (average, 2242; SD, 292) for H5 HA and 1779–2354 (average, 2027; SD, 221) for type A influenza. Thus, the cut-off indices were set at 3410 and 2911, respectively.

The sensitivity of the kit was compared with that of the ESPLINE® Influenza A&B-N (Fujirebio, Inc., Tokyo, Japan) kit, which is a commercially available rapid influenza diagnostic test (RIDT). This immunochromatography (IC)-based kit is meant to diagnose infection by all type A influenza viruses, including H5N1. However,

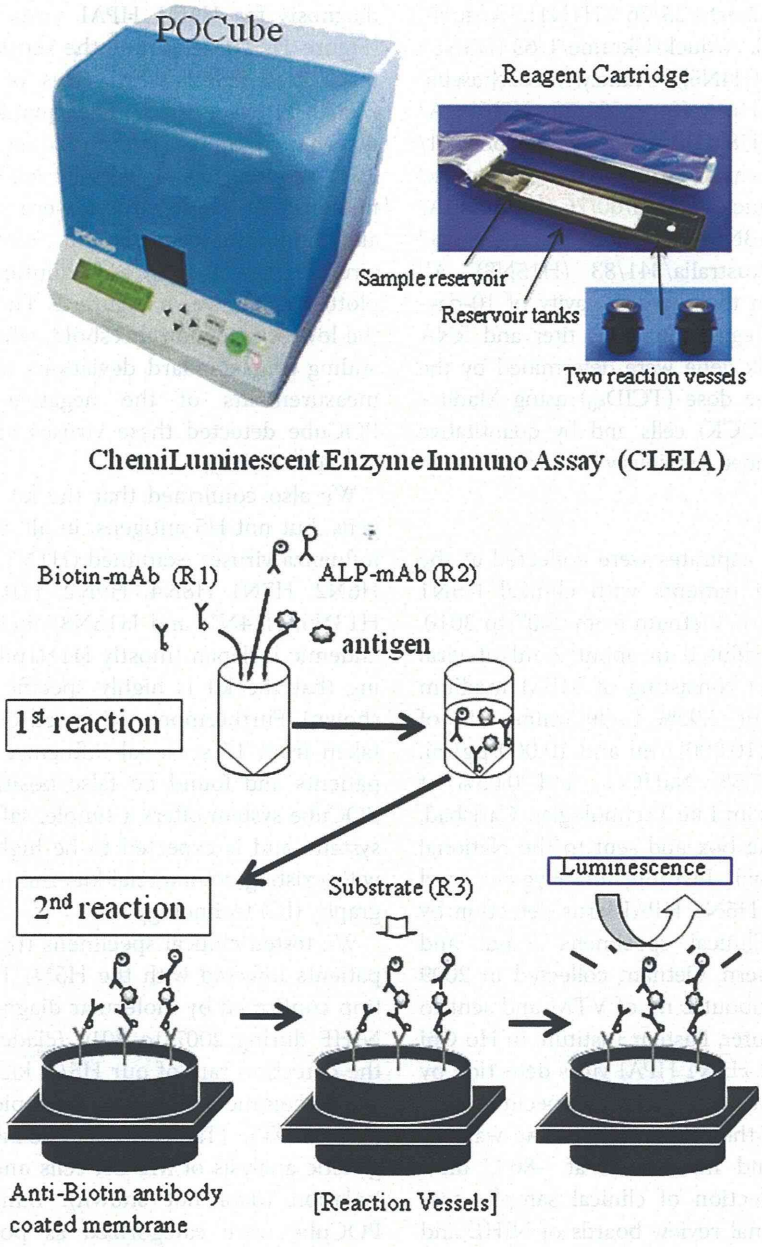

Figure 1 Principle of the POCube system. POCube is a fully automated chemiluminescence analyzer. In the H5/A kit, two Abs were incorporated, namely, one for H5 HA and the other for type A NP antigens of the influenza virus. One of the Abs is biotinylated and the other is conjugated to ALP. A sample is loaded into a reservoir tank in the cartridge, which is placed in the machine along with two reaction vessels. When the complex of antigen and two Abs is formed, it is trapped on an anti-biotin Ab-coated membrane in the reaction vessels. The membrane is then washed, a luminescent substrate for ALP is added, and the emitted luminescence is measured. Using the appropriate program, POCube automatically performs each of these steps, and the result is displayed as either positive or negative. Alternatively, the luminescence counts can be provided, if preferred.

because this kit uses an NP-specific antibody, it cannot discriminate H5N1 from other subtypes.

Virus preparation

The A/Vietnam/1194/2004 (NIBRG-14) virus, which has a modified HA gene and an NA gene derived from the HPAI A/Vietnam/1194/2004 (H5N1) virus within the backbone of six other internal genes of A/Puerto Rico/8/34 (PR8), as well as other modified H5N1 viruses, A/Indonesia/5/2005 (Indo5/PR-8-RG2), A/turkey/Turkey/1/2005 (NIBRG-23), and A/Anhui/01/2005 (Anhui01/PR8-RG5), were provided by the National Institute for Biological Standards and Controls (Potters Bar, UK). The following subtypes of influenza A viruses were also used to evaluate the specificity of the POCube H5/A kit and were obtained from the Influenza Virus Research Center

of the National Institute of Infectious Diseases (NIID), Tokyo, Japan: A/duck/Alberta/35/76 (H1N1), A/duck/Germany/1215/73 (H2N3), A/duck/Ukraine/1/63 (H3N8), A/duck/Czechoslovakia/56 (H4N6), A/turkey/Massachusetts/3740/65 (H6N2), A/duck/Hong Kong/301/78 (H7N1), A/turkey/Ontario/6118/68 (H8N4), A/turkey/Wisconsin/1/66 (H9N2), A/chicken/Germany/N/49 (H10N7), A/duck/England/56 (H11N6), A/duck/Alberta/60/76 (H12N5), A/gull/Maryland/704/77 (H13N6), A/mallard/Gurjev/263/82 (H14N5), and A/duck/Australia/341/83 (H15N8). All viruses were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs. The virus titer and RNA copy number of the matrix gene were determined by the 50% tissue culture infective dose ($TCID_{50}$) using Madin–Darby Canine Kidney (MDCK) cells and by quantitative real-time RT-PCR as described previously [18], respectively.

Clinical samples

Throat swabs or tracheal aspirates were collected at the point of admission from patients with clinical H5N1 virus infections in Northern Vietnam from 2007 to 2010. Clinical specimens were diluted in about 2 ml of viral transport medium (VTM) consisting of MEM medium supplemented with 1% of 2.92% L-glutamine, 1% of Penicillin- Streptomycin (10,000U/ml and 10,000 μg/ml, respectively), 1.5% of 37.5% $NaHCO_3$ and 0.05% of 250 μg/ml Fungizon (all from Life Technologies, Carlsbad, CA), packed into a cooling box and sent to the National Influenza Center, National Institute of Hygiene and Epidemiology (NIHE) for H5N1 HPAI virus detection by conventional RT-PCR. Clinical specimens (nasal and throat swabs) from Southern Vietnam collected in 2009 and 2012 were diluted in about 2 ml of VTM and sent to the National Influenza Center, Pasteur Institute in Ho Chi Minh City (PI-HCMC) for H5N1 HPAI virus detection by real-time RT-PCR. After a part of clinical specimen was used for RNA extraction, the remaining volume was aliquoted into cryotubes and maintained at −80°C until further analysis. The collection of clinical samples was approved by the institutional review boards of NIHE and PI-HCMC. All nasopharyngeal aspirates from patients, including seasonal and non-influenza, were collected after obtaining patient's written informed consent.

Results and discussion

Different combinations of anti-H5 HA mAbs [17] and a pair of biotinylated OM-b and ALP-conjugated 1C10 mAbs were selected for H5 HA detection.

The H5/A kit contains two sets of mAb mixtures to detect H5 HA simultaneously with NP antigens which are common to all influenza A viruses. This combination is important for the definitive diagnosis of H5 HPAI virus infection. When a clinical specimen is loaded into a reservoir tank in the cartridge, which is placed in the machine along with two reaction vessels, an automatic diagnosis for H5N1 HPAI virus infection is provided (Figure 1). To determine the sensitivity of the H5/A kit, inactivated H5N1 HPAI virus of representative clades (Table 1) were used. The quantities of viruses were determined by $TCID_{50}$ and quantitative real-time RT-PCR targeting the universally conserved matrix gene of influenza A. These viruses were serially 10-fold diluted and the luminescence counts were measured. Then, the virus $TCID_{50}$ titers at each dilution were calculated and plotted as shown in Figure 2. The dotted line indicates the lowest detection threshold, which was determined by adding four standard deviations to the average of eight measurements of the negative control. Thus, the POCube detected these viruses at a titer equivalent to 10^1–10^3 $TCID_{50}$.

We also confirmed that the kit detected type A antigens, but not H5 antigens, in all other subsets of type A influenza viruses examined (H1N1, H2N3, H3N8, H4N6, H6N2, H7N1 H8N4, H9N2, H10N7, H11N6, H12N5, H13N6, H14N5, and H15N8) including strains recently endemic in Japan (mostly H1N1pdm or type B), indicating that the kit is highly specific to H5 HA (data not shown). Furthermore, we tested clinical swab specimens taken from 15 seasonal influenza and 30 non-influenza patients and found no false positives of H5. Thus, the POCube system offers a simple, safe, and rapid diagnostic system, and is expected to be highly sensitive compared with existing commercial kits that use immunochromatography (IC) technology.

We tested clinical specimens (throat swabs) from nine patients infected with the H5N1 HPAI virus (identification confirmed by molecular diagnosis using RT-PCR) in NIHE during 2007 to 2010 (clade 2.3.4) and compared the detection rate of our H5/A kit by POCube with that of the commercially available rapid influenza diagnostic test (RIDT), (Table 2). The clade was confirmed by genetic analysis of MDCK cells and/or embryonated egg isolation (data not shown). Luminescence counts of POCube were categorized as positive or negative as described in the Methods. In 2007, clinical specimens were collected at two time points from patient A, whereas they were only collected once from each of the other patients (B–I). All the specimens were already

Table 1 H5N1 viruses used for titration

	Virus strain	Clade	$TCID_{50}$/50 μl (Log_{10})	RNA copies ($\times 10^{10}$/ml)
1	A/Vietnam/1194/2004 (NIBRG-14)	1.1	7.0	2.88
2	A/Indonesia/5/2005 (PR8-IBCDC)	2.1.3.2	7.4	3.81
3	A/turkey/Turkey/1/2005 (NIBRG-23)	2.2.1	5.9	1.60
4	A/Anhui/1/2005 (PR8-IBCDC RG-5)	2.3.4	8.3	3.73

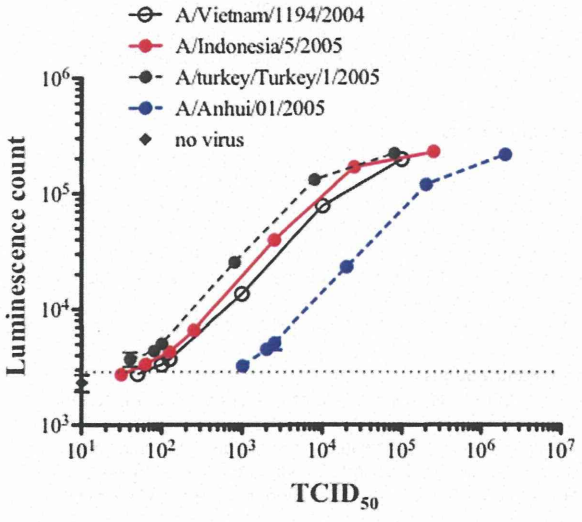

Figure 2 The sensitivity of the H5/A kit. H5N1 HPAI virus strains (Table 1) were serially diluted 10-fold, H5 HA antigens were measured using the POCube system, and the luminescence counts for each $TCID_{50}$ virus titer calculated according to each dilution were plotted. The dotted line indicates the lowest detection threshold, which was determined by adding four standard deviations to the average of eight measurements of the negative control.

diluted approximately 40-fold with VTM. We re-examined these clinical specimens for H5 HA RNA using conventional RT-PCR to assess the quality of the frozen samples: all were positive. One of these 10 samples (sample No. 9 of patient H) was H5-negative but type A-positive (Table 2), indicating that the abundance of viral particles was very low. One sample (No. 2 of patient A) was H5-positive but type A-negative. The quantity of viral particles was lower in this sample (No. 2) than in the sample (No. 1) that was collected at an earlier time point from the same patient (luminescence count of type A was 3,582 in sample No. 2 vs. 122,693 in sample No. 1). By contrast, a widely used commercially available RIDT based on IC using an anti-NP Ab showed very poor sensitivity, with only 10% of samples indicating a positive result. Furthermore, because the influenza NP is highly conserved, this kit cannot discriminate the A/H5N1 subtype from other influenza virus subtypes.

Table 2 Detection of H5N1 HPIA virus in clinical specimens in NIHE, Hanoi (clade 2.3.4)

Year	Patient ID	Test no.	POCube H5/A[1]		RIDT[2]
			H5 HA	A NP	
	A	1	+	+	+
		2	+	-	-
2007	B	3	+	+	-
	C	4	+	+	-
	D	5	+	+	+/–[3]
	E	6	+	+	-
2009	F	7	+	+	-
	G	8	+	+	-
	H	9	-	+	-
2010	I	10	+	+	-
% positive			90	90	10

[1]The POCube machine shows the results for H5 and type A viruses as "+" or "–" based on a predetermined cut-off index set (calculated as described in the "Methods").
[2]RIDT: commercial kit detecting universal NP antigen of influenza A virus (ESPLINE® Influenza A&B-N).
[3]Uncertain.

Table 3 Detection of H5N1 HPIA virus in clinical specimens in PI-HCMC (clade 1.1)

Year	Patient ID	Test no.	Specimen	POCube H5/A		RIDT
				H5 HA	A NP	
2009	J	11	Throat	+	+	-
		12	Throat	+	+	-
		13	Nasal	+	+	-
		14	Serum	+	+	-
2012	K	15	Throat	+	+	+
	L	16	Throat	-	-	-
		17	Nasal	+	+	-
	M	18	Throat	+	-	-
		19	Nasal	+	+	-
% positive				88.9	77.8	11.1

The H5/A kit was also evaluated using nine clinical specimens collected from one patient (J) in 2009 and from three patients (K–M) in 2012 (Table 3). These specimens were stored at the PI-HCMC after H5N1 HPAI virus (clade 1.1) infection was confirmed by genetic analysis of MDCK cells (data not shown). In patient J, throat swabs taken at two time points, a nasal swab, and a serum sample were all H5-positive and had similar luminescence counts of 5,282, 5,358, 5,344, and 6,448, respectively. In patient L, a throat swab was H5- and type A-negative, whereas a nasal swab was positive for both. In patient M, a throat swab was H5-positive and type A-negative, whereas a nasal swab was H5- and type A-positive. These results suggest that secretion of the

Table 4 Summary of H5N1 HPIA virus detection in clinical specimens from Vietnam

H5N1	POCube H5/A				% positive by POCube H5/A kit	% positive by RIDT
	Positive	Negative	Suspicious			
	+/+	–/–	+/–	–/+		
Clade 2.3.4	8	0	1	1	80.0	10
Clade 1.1	7	1	1	0	77.8	11.1
Total	15	1	2	1	78.9	10.5

virus is more abundant in the nasal mucosa than in the throat, and are not consistent with previous reports [19,20]. Also, the timing for sampling may affect the detection efficiency. However, because the number of samples tested in this study was very small, further testing is needed. The findings indicate that when H5N1 HPAI virus infection is suspected, it is important to collect samples from more than one anatomical location (i.e., throat and nose) and/or at more than one time point.

When samples are H5-positive but type A-negative, such as was the case for patient M (luminescence counts of H5 and type A were 4,071 and 2,622, respectively), the test needs to be repeated or confirmed by genetic analysis. This may occur when the level of virus antigen is at the threshold of detection, such as was the case for sample No. 2 of patient A. Again, the commercially available RIDT showed poor sensitivity, with only one of nine samples testing positive for type A.

Table 4 shows a summary of the results of the POCube H5/A kit test in samples collected from Vietnam, which detected 77.8% and 80.0% of clade 1.1 and clade 2.3.4 H5N1 HPAI virus infections, respectively. In these samples, the POCube test detected 78.9% of H5N1 HPAI virus infections, whereas the RIDT detected only 10.5%. This was probably because POCube is a highly sensitive chemiluminescence detection system that uses mAbs with a high affinity to H5 HA and also analyzes a much larger sample volume than the RIDT (up to 180 μl for POCube vs. about 5 μl for RIDT). Considering that only one in 19 samples tested negative for both H5 and A antigens, the POCube H5/A kit is highly useful to detect H5N1 HPAI virus infections in clinical settings. In this study, samples were diluted in VTM (about 2 ml); however, we believe the detection rate will improved even further if clinical swabs are tested with a reduced volume of this medium.

H5 HA HPAI virus detection using POCube and the H5/A kit is a highly sensitive and simple rapid diagnostic system that can be completed within 15 minutes. During the preparation of this manuscript, Sakurai et al. reported a 10–100-fold more sensitive detection of H5 HA using fluorescent beads and an improved IC method [21]; however, the clinical effectiveness of this method remains to be evaluated. We recently determined the epitopes of our anti-H5HA mAbs and found that OM-b, one of mAbs used for the H5/A kit, was broadly reactive to various clades of H5N1 influenza virus isolates in Asia, indicating that ther kit is quite useful for the diagnosis of H5N1 infection in Asian counties [22].

Conclusions

The POCube system using the H5/A kit is useful for the rapid detection of H5N1 HPAI virus infections in humans in hospitals and other clinical settings where technical resources are limited.

Abbreviations

HA: Hemmagglutinin; NA: Neuraminidase; NP: Nucleoprotein; HPAI: Highly pathogenic avian influenza; ALP: Alkaline phosphatase; RIDT: Rapid influenza diagnostic test; $TCID_{50}$: 50% tissue culture infectious dose; VTM: Viral transport medium.

Competing interests

KN and SM are research staffs in Toyobo Co.Ltd. and collaborated with YYY, KO and TK in NIID (a government research institute) for this study, which was formally supported by a grant from the Health Science Foundation of the Ministry of Health, Labor and Welfare of Japan (KHC1218). The patent of H5/A kit was filed by Toyobo Co. Ltd and NIID. This may not cause any competing non-financial interests were they to become public after the publication of this manuscript.
All other authors declare no potential conflicts of interest.

Authors' contributions

YTY, KN, SM and KT determined the protocol for the assay. HLKN, MTQL, GTD, LTN prepared clinical specimens and helped YTY and KT for the analysis. MKI, HT and HT participated in the design of the study and helped to draft the manuscript. KO, SI and MT helped to analyze the results and finalize the manuscript. All authors read and approved the final manuscript.

Authors' information

YTY worked in NIID for this study and recently moved to the Department of Medical Technology, School of Human Sciences, Tokyo University of Technology, 5-23-22 Nishi-Kamata, Ohta-ku, Tokyo 144–8535, Japan.

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Broad Cross-Reactive Epitopes of the H5N1 Influenza Virus Identified by Murine Antibodies against the A/Vietnam/1194/2004 Hemagglutinin

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Abstract

There is an urgent need for a rapid diagnostic system to detect the H5 subtype of the influenza A virus. We previously developed monoclonal antibodies (mAbs) against the H5 hemagglutinin (HA) for use in a rapid diagnostic kit. In this study, we determined the epitopes of the anti-H5 HA murine mAbs OM-b, AY-2C2, and YH-1A1. Binding assays of the mAbs to different strains of H5 HAs indicated that OM-b and AY-2C2 cross-reacted with HAs from clades 1, 2.1.3.2, 2.2, and 2.3.4, whereas YH-1A1 failed to bind to those of clades 2.1.3.2 and 2.3.4. HA chimeras revealed that the epitopes for each of the mAbs were in the HA1 region. Analysis of escape mutants revealed that OM-b and AY-2C2 mAbs interacted mainly with amino acid residues D43 and G46, and the YH-1A1 mAb interacted with G139 and K or R140 of H5 HA. Multiple alignments of H5 HA protein sequences showed that D43 and G46 were very conserved among H5N1 HAs, except those in clade 2.2.1 and clade 7 (88.7%). The epitope for YH-1A1 mAb was highly variable in the HAs of H5N1, although it was well conserved in those of H5N2-N9. The OM-b and AY-2C2 mAbs could bind to the HAs of clades 1.1 and 2.3.2.1 that are currently epidemic in Asia, and we conclude that these would be effective for the detection of H5N1 infections in this region.

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Introduction

The H5N1 influenza virus is a global threat to birds and humans, and by January 2014, there had been 650 cases of infections in people, with 386 deaths [1]. The disease in humans is epidemic in Asian and African countries such as Vietnam, Indonesia, Cambodia, and Egypt. Infections by H5N1 in people are limited to those who had close contact with infected animals, although the range and severity of symptoms in humans is not clear. For example, meta-analysis of serological studies on human H5N1 infections indicates a large number of missed infections [2,3]. Several reports have highlighted outbreaks of human-adapted H5N1 viruses, although the level of risk has not been fully ascertained [4–8].

Rapid diagnosis of H5N1 infections is essential because patients treated in the early stages of the disease have a significantly lower level of mortality [9,10]. Human H5N1 infections are mostly diagnosed by RT-PCR, which requires a few hours and some expertise to obtain results. Rapid and simple systems for the immunological detection of viral antigens have also been developed; however, these kits can have a low sensitivity [11] and cross-reactivity with other subtypes [12,13]. The development of a rapid and reliable detection system for H5N1 without the need for RNA extraction would help to deliver an earlier clinical diagnosis in more localized areas.

For these reasons, several monoclonal antibodies (mAbs) that specifically recognize hemagglutinins (HAs) from the H5 subtype influenza viruses (H5 HA) were previously created in the development of a rapid detection system for H5N1 [14]. However, the range of cross-reactivity to H5 HAs is unclear because H5N1 viruses are still evolving and diversifying into multiple lineages, which are classified into clades (0–9) and subclades on the basis of their HA genealogy [15]. It is important to understand the epitope and cross-reactivity of anti-H5 HA mAbs in the development of a broadly reactive H5N1 influenza diagnostic kit.

In this study, we determined the epitopes of anti-H5 HA mAbs, and evaluated their range of reactivity to different clades of human H5N1 viruses. This was achieved by assessing the cross-clade reactivity of wild-type HAs, assessing the recognition sites of HA chimeras by flow cytometry, and analyzing escape mutants.

Materials and Methods

Viruses and Cells

A/Vietnam/1194/2004 (clade 1), A/Vietnam/1203/2005 (clade 1), A/Indonesia/05/2005 (clade 2.1.3.2), A/Turkey/12/

2006 (clade 2.2), and A/Anhui/01/2005 (clade 2.3.4) were provided by the National Institute of Biological Standards and Controls (NIBSC, UK). A/Vietnam/VP-12-03/2012 (clade 1.1) and A/Narita/1/2009 (H1N1) were isolated and provided by the National Influenza Center, Pasteur Institute, Vietnam, and the Influenza Virus Research Center (IRC), NIID, Japan, respectively. A/whooper swan/Hokkaido/4/2011 (clade 2.3.2.1) was provided by Hokkaido University [16]. Culturing of the infectious H5N1 virus was done in a biosafety level 3 (BSL3) facility at the IRC, NIID, Japan. Batches of 293T cells and Madin–Darby canine kidney (MDCK) cells were cultured in Dulbecco's Modified Eagle's Medium and Minimum Essential Medium (Invitrogen, Carlsbad, CA, US), respectively, supplemented with 10% fetal bovine serum and incubated in a 5% CO_2 atmosphere at 37°C.

Antibodies

The monoclonal antibodies (mAbs) OM-b, AY-2C2, and YH-1A1 were produced previously [14]. C179 mAb (TaKaRa, Japan) was used as a positive control [17], and mouse IgG1 (BD Biosciences, San Diego, CA) and IgG2a (mAb Nk1.1) [18,19] were used as isotype controls for flow cytometry analyses.

Expression Vectors

Total RNA was extracted from virus stocks, and the HA genes were amplified by RT-PCR using the following primers: RT primer (Uni12), 5′-AGCAAAAGCAGG-3′; PCR primer set: 5′-GTCGACATGGAGAAAATAGTGCTTCTTTTTGCA-3′ and 5′-GTCGACATGGAGAAAATAGTGCTTC-3′. The HA gene fragments were cloned into pIRES-hrGFP-1α (Agilent) or a modified pENTR11 vector (Invitrogen, Carlsbad, CA) that contains the *Tight* promoter from pRetroX-Tight Pur (Clontech, USA) upstream of the multi-cloning site (MCS) and IRES-hrGFP sequences from pIRES-hrGFP-1α downstream of the MCS. The cloned pENTR11 was then recombined into the pCSII-RfA-Ed vector using the Gateway system (Invitrogen). pCSII-RfA-Ed was generated by replacing the EGFP gene of pCSII-RfA-EG (provided by Dr. Miyoshi, RIKEN, Japan) with the DsRed-express gene as follows: KpnI (blunted)/NotI fragment of pDsRed-Express vector (BD Biosciences) was subcloned into XhoI (blunted)/NotI site of pCSII-EF-MCS (provided by Dr. Miyoshi). Then, ApaI fragment of the pCSII-EF-MCS containing the DsRed-express gene was inserted into ApaI sites of pCSII-RfA-EG.

pIRES-hrGFP-1α vectors harboring chimeric or escaped mutant HAs were generated by site-directed mutagenesis (KOD Plus Mutagenesis Kit, Toyobo, Japan) [20]. For domain swapping, the globular or HA2 regions were PCR amplified from the pIRES-hrGFP-1αvector harboring A/Vietnam/1194/2004 HA or A/Narita/1/2009 HA (mega-primers). Then the PCR product was used as a mega-primer to produce the chimeric HA vector according to the mega-priming method of the site-directed mutagenesis [21]. pIRES-hrGFP-1α vectors harboring the chimeric HA gene were subcloned into pCSII-RfA-Ed as described above. The pRetroX-Tet-Off Advanced vector (Clontech) was used to express a tetracycline-controlled transactivator to activate the Tight promoter-transcriptions [22].

Overexpression of HA Proteins and Flow Cytometry

HA-expressing vectors were transfected or co-transfected with pRetroX-Tet-Off Advanced into 293T cells using Lipofectamine 2000. Cells were collected 24 h post-transfection (hpt) and mixed with anti-H5 mAbs (30 μg/mL) or C179 mAb (5 μg/mL). Cells were mixed with Alexa Flour 647-conjugated anti-mouse immunoglobulin (Jackson ImmunoResearch, West Grove, PA) and incubated on ice for 10 min, and then analyzed using a FACS Calibur flow cytometer (BD Bioscience).

Western Blotting

Cells expressing HAs were lysed with RIPA buffer (10 mM Tris-HCl (pH 7.6), 1% Triton X-100, 1% sodium-deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA (pH 8.0)) supplemented with a protease inhibitor cocktail (Roche, Switzerland). Lysates were separated by SDS-PAGE under reducing conditions, and then transferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked with Tris-buffered saline containing 0.05% Tween-20 and 0.3% skimmed milk, and then reacted for 1 hour with a mixture of sheep anti-H5 and H1 HA sera (anti-VN1194 HA and anti-A/New Caledonia/20/99 (IVR-116) HA sera, provided by IRC, NIID, Japan). The membrane was then incubated for 30 min with HRP-conjugated anti-goat/sheep immunoglobulin or anti-mouse immunoglobulin antibodies (Jackson ImmunoResearch).

Selection of Escape Mutant Viruses

The A/Vietnam/1194/2004 vaccine strain virus (10^6 $TCID_{50}$/50 μL) was separately incubated with each mAb (OM-b, 0.625 μg/mL; AY-2C2, 0.313 μg/mL; YH-1A1, 0.078 μg/mL) for 30 min at 37°C. Mixtures were serially diluted and used to infect MDCK cell layers in a 96-well microtiter plate. At 5–7 days post-infection, the supernatants of cells with cytopathic effects were harvested. The procedure was repeated twice and the final supernatants were then added to MDCK cells in a 6-well plate. Escape mutants were isolated using a plaque assay as described previously [23]. Total RNA was extracted from the escape mutants and used as the template for RT-PCR amplification of the HA gene using the primers described in the Expression vectors subsection. The nucleotide sequences of the HA gene PCR products were analyzed by direct sequencing.

Neutralization Titers of mAbs with Escape Mutant Viruses

A confluent monolayer of MDCK cells was prepared in each well of a 96-well microtiter plate. Each of the mAbs were mixed with 100 $TCID_{50}$ of the escape mutant virus in the presence of trypsin and incubated at 37°C for 30 min [24]. Suspensions of the viruses were individually added to the MDCK cells and incubated for 7 days at 34°C in 5% CO_2. Cells were then fixed with 10% formaldehyde, and stained with NB solution (0.1% naphthol blue black, 0.1% sodium acetate, and 9% acetic acid) before measuring OD_{630}. Cell viability was calculated using a calibration of the OD_{630} values from uninfected and virus-only wells as 100% and 0%, respectively. The concentration of the final dilution that reduced the cytopathic effects of the virus by 50% was taken as the neutralizing titer.

In silico Analysis of HA Structures

The crystal structures of VN1194 HA (PDB ID: 2IBX) and H1pdm HA (PDB ID: 3LZG) were analyzed by *in silico* modeling, and root-mean-square deviation (RMSD) measurements [25] were determined using *Molecular Operating Environment (MOE)* ver. 2013.08 software (Chemical Computing Group Inc., Canada). To model chimeric HAs, each domain of H5 HA was grafted onto that of H1pdm HA with a minimal energy conformation.

Statistics

Experiments were independently conducted three times. The data was analyzed using a Student's t-test and p-values of <0.05

were considered statistically significant. Error bars represent standard deviations.

Results

Cross-clade Reactivities of H5N1 HAs

Anti-H5 HA mAbs were produced by immunizing BALB/c mice with inactivated VN1194 viruses [14]. The characteristics of OM-b, AY-2C2, and YH-1A1 mAbs used in this study are shown in Table 1. Each mAb had micro-neutralization (MN) activity but no hemagglutination inhibition (HI). It was previously proposed that the AY-2C2 mAb recognizes a conformational epitope [14].

The binding of anti-H5 HA mAbs to different clades of H5 HAs was determined using a flow cytometer to understand the breadth of cross-reactivity. The HA genes from five representative H5N1 strains were cloned into expression vectors and transfected into 293T cells. Western blot analysis of HA-expressing cells using a mixture of polyclonal anti-H5 and anti-H1 HA sera revealed that precursor HA0 was present in all transfectants. Cleaved HA1 and HA2 proteins were detected in H5 HA transfectants, although clade 2.3.4 HA showed weak cleavage activity (Figure 1A), and is consistent with the findings of Tang *et al.* [26].

These transfectants were reacted with the anti-H5 HA (OM-b, AY-2C2, and YH-1A1) mAbs and analyzed by flow cytometry. The ratio of the mean fluorescence intensity (MFI) of the mAb relative to that of the C179 mAb, which broadly recognizes HAs of group 1 influenza viruses, was determined to compare the binding affinity of each transfectant (Figure 1B). Isotype mAbs were used as controls for background staining. YH-1A1 mAb bound to clade 1 and 2.2 HAs, but not to clade 2.1.3.2 or 2.3.4 HAs. OM-b and AY-2C2 mAbs bound to HAs from all clades. YH-1A1 mAb bound strongly to clade 2.2 HA, but weakly to the clade 1 (VN1194) HA that is used for immunization. Of the mAbs, OM-b bound the strongest to clade 2.3.4 HA. The results suggest that OM-b and AY-2C2 mAbs recognize the well-conserved regions of H5 HA, whereas YH-1A1 mAb interacts with variable regions.

Mapping of the H5 HA Domain Recognized by anti-H5 HA mAbs

Three plasmids expressing chimeric HAs were constructed by domain swapping between the VN1194 HA and H1pdm HA (Figure 2A) to identify the precise binding regions of the mAbs. The H5/1 chimera has amino acid sequences corresponding to that of VN1194 HA in the HA1 region and that of H1pdm HA in the HA2 region. The H1/5 chimera has H1pdm HA1 and VN1194 HA2 regions. The globH5 chimera has a VN1194 globular head region and other regions derived from H1pdm HA (amino acids 42–275 of H1pdm HA replaced with amino acids 42–274 of VN1194 HA, GenBank: ABP51976 and ACR09396, respectively). Computer simulation revealed that the conformation of the HA1 stem loop region of the H5/1 chimera was different to that of wild-type H5 HA (Figure 2B and Figure S1).

The RMSD of Cα indicated that the globular head region of the chimera was similar to that of wild-type H5 HA (RMSD = 1.606 angstrom (Å)). The H1/5 chimera maintained the conformation of the stem and globular head regions corresponding to the original H5 and H1 subtypes, respectively (RMSD = 0.488 Å). The globH5 chimera also appeared to maintain conformation of the original H5 and H1 HAs, except around the boundary region between the globular head and stem (RMSD of the boundary region = 2.866 Å, and RMSD of the other region = 1.672 Å).

Plasmids of the chimeric HAs were transfected into 293T cells, and their expression was analyzed by Western blotting using a mixture of polyclonal goat sera against H5 and H1 HA (Figure 2C). Consistent with the wild-type VN1194, H5/1 maintained cleavage activity without trypsin treatment. This was because the cleavage site was derived from the VN1194 HA (Figure 2A). The binding of mAbs to each HA chimera was assessed by flow cytometry (Figure 2D), and the results are summarized in Table 2. C179 mAb bound to the globH5 and H1/5 chimeras, but not to H5/1. OM-b, AY-2C2, and YH-1A1 mAbs interacted well with the H5/1 chimera, but were unable to bind to H1/5, confirming their recognition of the HA1 domain. These three mAbs also bound to the globH5 chimera; the strength of attachment was greater for YH-1A1 than for OM-b and AY-2C2. Thus, the results indicate that the mAbs recognize the globular region of the H5 HA with different affinities.

Mab Binding Sites

Escape mutant viruses were selected after a second round of infection of VN1194 vaccine strains in the presence of each mAb to deduce which amino acid positions may contribute to the interaction with H5 HA. Results of nucleotide sequencing of the HA coding region are shown in Table 3. At amino acid position 43 in the escape mutants against AY-2C2 mAb, substitutions of D to N and D to Y were found at a similar frequency. The same amino acid substitutions at D43N and D43Y were present in the escape mutants against OM-b mAb. D45Y and G46E substitutions were also observed in escape mutants of OM-b mAb. The escape mutants against YH-1A1 mAb harbored G139E or K140E substitutions.

The MN activity of each mAb to the escape mutants was determined (Table 4). The escape mutants of OM-b and AY-2C2 were resistant to neutralization by OM-b and AY-2C2 mAbs, but not by YH-1A1 mAb. However, resistance of the AY-2C2 escape mutants against the OM-b and AY-2C2 mAbs was weak. By contrast, the escape mutants of YH-1A1 mAb became resistant to neutralization only by YH-1A1 mAb. The results shown in Tables 3 and 4 indicate that OM-b and AY-2C2 have major epitopes at D43, D45, and G46. YH-1A1 has major epitopes at G139 and K140.

The three-dimensional structures of the mutation sites of H5 HA were analyzed using the MOE program. Epitope mapping of H5 HA (PDB ID: 2IBX) revealed mutations at the origin of the

Table 1. Characteristics of anti-HA mAbs.

mAb	JH gene	Subclass*	HI activity	MN activity (μg/50 μL)*	Clade dependence*
OM-b	4	G2a	-	0.625	No
AY-2C2	2	G1	-*	0.313	No
YH-1A1	4	G2a	-*	0.078	Yes

*Results reported previously by Ohnishi *et al.* [14].

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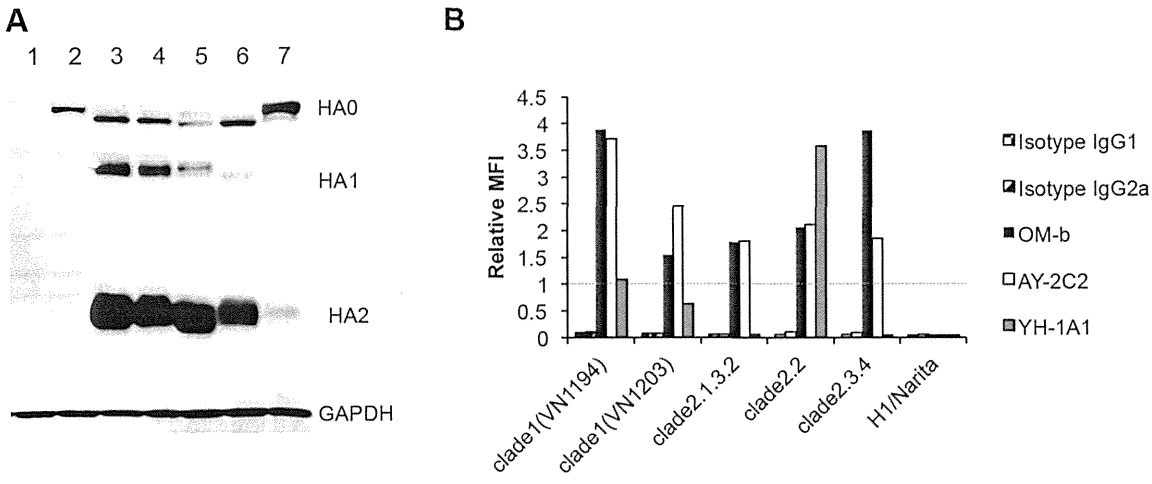

Figure 1. Cross-clade binding activities of the four mAbs. (A) Expression patterns of HA proteins. H5 HA expression vectors were transfected into 293T cells. Cells were collected at 24 hpt and analyzed by Western blotting. Sheep anti-H5 HA and H1 HA polyclonal sera were used to detect HAs (upper panel). Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression are shown as a loading control (lower panel). Cells transfected with an empty vector (lane 1) and the expression vectors of H1pdm HA (lane 2), VN1194 HA (lane 3, clade 1), VN1203 HA (lane 4, clade 1), clade 2.1.3.2 HA (lane 5), clade 2.2 HA (lane 6), and clade 2.3.4 HA (lane 7) were analyzed. (B) Interactions between the H5 HAs and mAbs. The cells described in (A) were incubated with mAbs and the reactivity was determined by flow cytometry. The MFI of each mAb was normalized against that of mAb C179 (1.0) to account for different expression levels. A representative result of three independent experiments with high reproducibility is shown.
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globular head (Figure 3A and B, red). The YH-1A1 mutations were located on the surface of the globular domain a short distance from the receptor-binding site (Figure 3A and B, blue).

The escape mutant HAs (D43N, D45Y, G46E, G139E, and K140E) were overexpressed in 293T cells and analyzed by flow cytometry (Figure 3C and 3D) to determine whether conformational changes affect the binding sites of mAbs. Isotype IgG1 or IgG2a was used as a background control (data not shown). OM-b and AY-2C2 mAbs could not bind with the G46E and D43N mutants (Figure 3C). However, there was no significant change in the binding activities between the D45Y mutant and OM-b or AY-2C2 mAbs. YH-1A1 mAb did not interact with the G139E or K140E mutant HAs (Figure 3D).

The computer simulation revealed that binding of the different mAbs was affected by altering the physicochemical status of the H5 HA molecule. A shift in the charge from negative to positive at the D43N mutation site occurred without altering the size of the side chains (Figure 3E) [27]. The G46E and G139E mutations appear to change the surface structure at this particular site (Figure 3F for G46E and data not shown for G139E). The K140E mutation appeared to change the charge from positive to negative, and also affected the shape of the surface (data not shown).

The results of this study revealed that the binding sites of OM-b and AY-2C2 mAbs overlapped and were located at D43 and G46, whereas those of YH-1A1 mAb were located at G139 and K140.

Epitope Conservation among H5 Influenza Viruses

Epitope sequences of the escape mutants were compared with H5N1 strains (Table 5). The D43, D45, and G46 epitopes of OM-b and AY-2C2 mAbs, and the G139 epitope of YH-1A1 mAb, were conserved among all the clades. The K140 epitope of YH-1A1 was variable. YH-1A1 mAb interacted with clade 1 and clade 2.2 (K140 and R140, respectively), but not with clade 2.1.3.2 and 2.3.4 HAs, as shown in Figure 1B. YH-1A1 may preferentially bind to a positively charged amino acid at the 140th position, but not to one that is neutrally or negatively charged, such as serine, threonine, and glutamine acid.

The ability of OM-b and AY-2C2 mAbs to recognize H5N1 strains that are currently epidemic in humans and birds (i.e., clades 1.1 and 2.3.2.1) was determined. A/Vietnam/VP-12-03/2012 (clade 1.1) maintains the OM-b and AY-2C2 epitope sequences, and A/whooper swan/Hokkaido/4/2011 (clade 2.3.2.1) harbors a D45N substitution. These HAs were expressed in 293T cells, and attachment of the OM-b and AY-2C2 mAbs to the HAs was determined by flow cytometry. OM-b and AY-2C2 mAbs could bind to the clade 1.1 and 2.3.2.1 HAs (Figure 4). The results indicate that D43 and G46, but not D45, are important for recognition by OM-b and AY-2C2 mAb, and that these mAbs could be used to detect current H5N1.

Alignments of influenza HA amino acid sequences from the Influenza Virus Resource database [28] showed that the conservation rates of the epitope sites among human and avian H5N1 strains were 88.8% (D43), 99.9% (G46), 99.2% (G139), and 61.0% (K140 or R140) (Table 5). The overall epitope conservation rates for H5 influenza viruses in the database are estimated in Table 6. The conservation rate of human and avian H5N1 in the OM-b and AY-2C2 epitope sequences (D43 and G46) was 88.7% and 60.6% for YH-1A1 (G139 and K or R140). By contrast, the conservation rate of OM-b and AY-2C2 epitope sequences among avian H5N2-N9 strains was as low as 0.74%. This may be because the amino acid sequences of H5N2-N9 HAs, except H5N5, contain serine at the 43rd position, which appears to change the electrostatic potential in a manner similar to the D43N mutation. Alternatively, the YH-1A1 epitope sequences are highly conserved (94.0%), with most of the H5N2-N9 HA sequences containing glycine at the 139th position and arginine at the 140th position. These results suggest that OM-b and AY-2C2 mAbs can specifically detect H5N1 infections.

The results also revealed that the OM-b and AY-2C2 epitope was conserved among 85.5% of human H5N1 strains (Table 6). The remaining 14.5% of H5N1s correspond to one strain in clade 2.1.3.2 that harbors a G46R substitution (A/Indonesia/CDC582/2006), and the majority of clade 2.2.1 that are currently epidemic in Egypt and contain D43N substitutions [29,30]. We consider

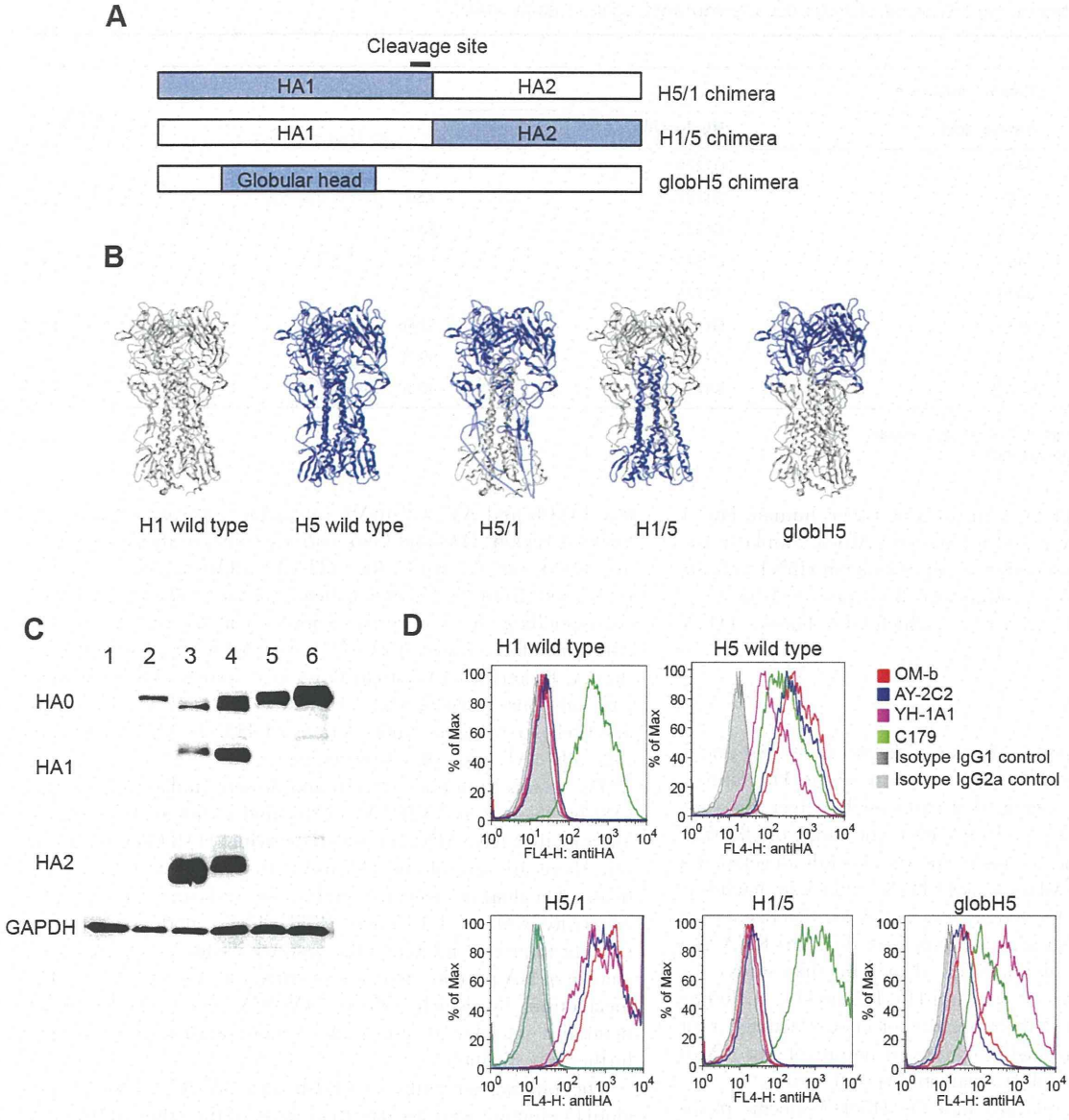

Figure 2. Epitope screening with HA chimeras. (A) Schematic diagram of chimeric HAs. A series of chimeric HAs were generated by domain swapping between VN1194 HA (blue) and H1pdm HA. (B) Formations of chimeric HAs simulated by MOE based on the crystal structure of VN1194 HA (PDB ID: 2IBX, blue) and H1pdm HA (PDB ID: 3LZG, gray). (C) Expression of the chimeric HAs in mammalian cells. The chimeric HA expression vectors were co-transfected with pRetroX-Tet-Off Advanced into 293T cells. Cells were collected at 24 hpt and analyzed by Western blotting. Upper panel, expression patterns of the HAs; lower panel, loading control (GAPDH). Cells transfected with an empty vector (lane 1) and the expression vectors of H1pdm HA (lane 2), VN1194 HA (lane 3), H5/1 chimera (lane 4), H1/5 chimera (lane 5), and globH5 chimera (lane 6) were analyzed. (D) Interaction between the chimeric HAs and OM-b, AY-2C2 and YH-1A1mAbs. Cells described in (B) were incubated with mAbs, and the reactivity was analyzed by flow cytometry. Histograms of levels of reactivity against each mAbs in cells expressing the indicating HAs.
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Table 2. Summary of the binding of mAbs to chimeric HAs.

HA	OM-b	AY-2C2	YH-1A1	C179
H5 HA	++	++	+	++
H1 HA	–	–	–	++
H5/1 chimera	++	++	++	–
H1/5 chimera	–	–	–	++
globH5 chimera	+	+	++	++

Differences in the MFIs to HAs between the mAbs and its isotype control were categorized as follows: over 300 (++), between 20 and 300 (+), and under 20 (–).
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Table 3. Mutation sites in the HA gene of virus escape mutants against each mAb.

mAb	Escape mutation		Frequency (%)
	Amino acid	Nucleotide	
OM-b	D43N	G127A	83.32
	D43Y	G127T	5.56
	D45Y	G133T	5.56
	G46E	G137A	5.56
AY-2C2	D43N	G127A	57.14
	D43Y	G127T	42.86
YH-1A1	G139E	G416A	60.00
	K140E	A418G	40.00

The mutations occurred singly in each escape mutant.
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that the OM-b and AY-2C2 mAbs will cover human H5N1 infections emergent in Asia, but not those in Africa. Similarly, the results of the epitope conservation analysis in avian H5N1 indicate that the mAbs cannot cover sublineage B viruses of clade 2.2.1, and clade 7, 7.1, and 7.2 viruses, which also harbor D43N substitutions.

Discussion

There is an urgent need to develop a diagnostic system that is highly sensitive and rapid for the detection of the H5 subtype influenza virus. Several systems that can rapidly detect this virus were recently developed [31,32]; however, the range of effectiveness for these approaches is unknown. We recently developed a chemiluminescent ELISA-based rapid H5N1 influenza diagnostic system using OM-b mAb and demonstrated its effectiveness at screening for the infection prior to analysis of the virus RNA at a centralized laboratory (unpublished data). In this study, we determined the epitopes of our anti-H5 HA mAbs, including OM-b, and characterized their breadth of cross-reactivity. Our findings suggest that the highly conserved nature of the OM-b epitope make it ideal to detect Asian strains of the virus.

Analysis of chimeras revealed that the H5 HA-specific mAbs interact with the HA1 of H5 HA. Escape mutant analysis showed that OM-b and AY-2C2 mAbs interacted with the origin of the globular region, D43 and G46, corresponding to the antigenicity-associated site C, while the YH-1A1 mAb reacted with the protrusion from the globular head surface, G139 and K140, corresponding to the antigenicity-associated site A [33,34]. Antigenic drift events of H5N1 favor the residues in antigenic sites A, B, and D rather than those in C and E [33]. Consistent with this, our results also showed that OM-b and AY-2C2 mAbs are likely to recognize more strains of H5N1 (88.7% coverage) than YH-1A1 mAb (60.6% coverage).

The results from our previous and present studies indicate that OM-b, AY-2C2 and YH-1A1 mAbs bind to the globular region, and that they have MN, but not HI, activities [14]. Consistent with this, there are several anti-HA mAbs that attach to the globular head other than the receptor-binding site and can neutralize virus infectivity without HI activity [35]. Their mode of action is thought to involve inhibiting the post-acidification conformational change of HA proteins, which is essential for virus entry [27]. The mechanism by which OM-b, AY-2C2 and YH-1A1 mAbs neutralize infection by the virus remains unclear and warrants further investigation.

The binding strengths of OM-b and AY-2C2 mAbs to the globH5 chimera were weaker than those of the other mAbs. This may be because the epitope is located close to the site of exchange.

Table 4. Neutralization titers of mAbs with escape mutants of the VN1194 virus.

Escape mutant		MN activity*		
mAb	Mutation	OM-b	AY-2C2	YH-1A1
OM-b	D43N	−	−	+
	D43Y	−	−	±
	D45Y	−	−	+
	G46E	−	−	+
AY-2C2	D43N	±	±	+
	D43Y	±	±	+
YH-1A1	G139E	+	+	−
	K140E	+	+	−

*Differences between the micro-neutralization (MN) titers of mAbs in reactions with the wild-type VN1194 virus and its escape mutants. +, MN titer of the mAb does not differ from that with the wild-type virus; ±, MN titer is 2-8-fold greater than that with the wild-type virus; −, MN titer is at least 16-fold greater than that with the wild-type virus.
doi:10.1371/journal.pone.0099201.t004

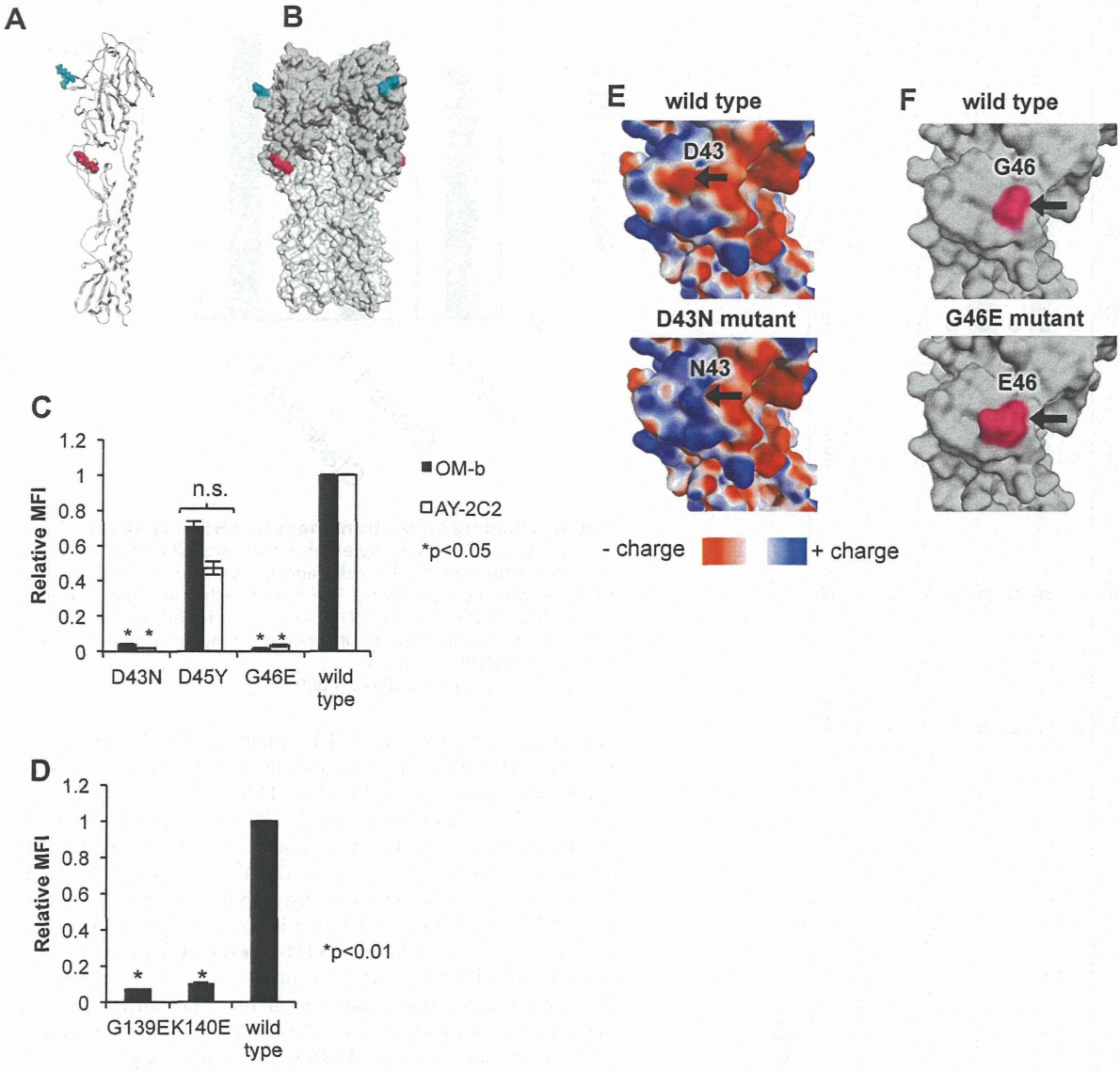

Figure 3. Binding of the mAbs to escape mutant HAs. (A and B) Structures of the mutation sites. Amino acid mutation sites are mapped onto the ribbon structure of the H5 HA monomer (A) and a surface representation of trimeric H5 HA (B). The globular region is colored dark gray. Sites of escape mutations against AY-2C2 and OM-b mAbs are colored red, and those against YH-1A1 mAb are colored blue. (C and D) Results of flow cytometry. A series of pIRES-hrGFP-1α vectors expressing the mutant HA were transfected into 293T cells. At 24 hpt, the cells were collected and incubated with the OM-b (C), AY-2C2 (C), or YH-1A1 (D) mAbs, and the binding activities were determined by flow cytometry. The MFI of each mAb was normalized against that of the C179 mAb to account for different expression levels. The relative MFI of each mAb to that of the wild-type HA is shown. P-values were calculated by Student's t-test. No significant differences with the wild type is denoted by n.s. Standard deviation is represented by vertical bars. (E) Surface of the D43N mutant. Surface maps of wild-type VN1194 HA (upper panel) and D43N mutant HA (lower panel) are shown. The mutant formation was simulated by MOE based on the crystal structure of the VN1194 HA monomer (orange, negatively charged area; white, neutral area; blue, positively charged area). (F) Surface of the G46E mutant. Surface maps of wild-type VN1194 HA (upper panel) and G46E mutant HA (lower panel) are shown. (G46 and E46 are shown in red).
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Computer simulation of the structure indicated that the ruggedness and charge of the HA surface, particularly around the epitope sites, differ between globH5 and wild-type H5 HA (data not shown). The widely cross-reactive C179 mAb did not bind to the H5/1 chimera, despite the presence of the C179 mAb epitope [17,36]. This may have a structural explanation because the folding pattern of the HA1 stem loop of the H5/1 chimera was notably different from that of the wild-type HA (Figure 2B and Figure S1). An increased distance of the main epitope sites of the C179 mAb could result in poorer binding to the H5/1 chimera (distances between T318 of HA1 and V52 of HA2 are 22.74Å in H5/1 HA, and 11.04Å in the wild-type H5 HA).

D43 and G46 affect the affinities of OM-b and AY-2C2 mAbs, although the frequency of the escaped mutant virus at G46 was low or not isolated. G46 may be an important location that governs the structure and function of the HA, and accordingly, it may be difficult for a mutation to occur at that site. This may explain why the OM-b and AY-2C2 epitopes are highly conserved among human and avian H5N1 (99.9% identity).

Despite the inability of the OM-b and AY-2C2 mAbs to neutralize the D45Y virus, they were able to bind with the D45Y

Table 5. Alignments of the epitope sequences among H5N1 strains.

Interacting antibody		OM-b/AY-2C2 epitope*			YH-1A1 epitope*	
Amino acid sequence (H5 numbering)		**43**	**45**	**46**	**139**	**140**
	A/Vietnam/1194/2004 (1)	D	D	G	G	K
	A/Vietnam/1203/2004 (1)	D	D	G	G	K
Human	A/Indonesia/05/2005 (2.1.3.2)	D	D	G	G	S
strain	A/Turkey/12/2006 (2.2)	D	D	G	G	R
(clade)	A/Anhui/01/2005 (2.3.4)	D	D	G	G	T
	A/Vietnam/VP-12-03/2012 (1.1)	D	D	G	G	Q
	Escape mutants	N	Y	E	E	E
Avian strain (clade)	A/whooper swan/Hokkaido/4/2011 (2.3.2.1)	D	N	G	G	N
Conservation rate among human and avian H5N1 (%)		**88.8**	**87.4**	**99.9**	**99.2**	**60.8**

*Amino acids in italic letters are critical changes for OM-b/AY-2C2 or YH-1A1 binding.
doi:10.1371/journal.pone.0099201.t005

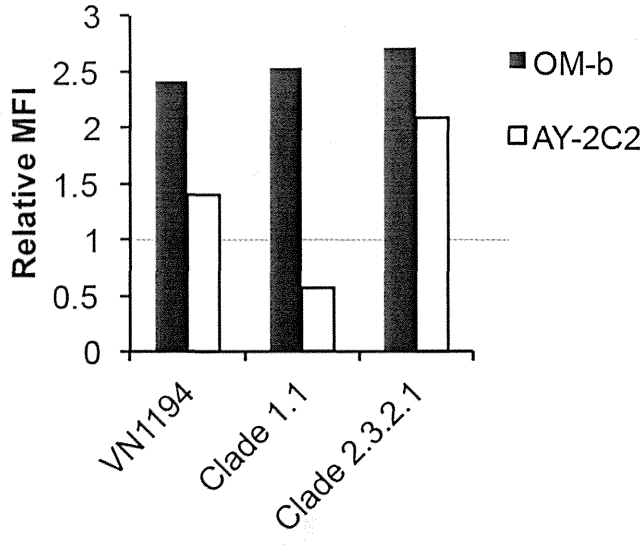

Figure 4. Binding of HAs from the recent H5N1 epidemic. Clades 1.1 and 2.3.2.1 HA genes were subcloned into pIRES-hrGFP-1α. HAs were overexpressed in 293T cells, and the binding of the mAbs was determined by flow cytometry. The MFI of each mAb was normalized against that of the C179 mAb (1.0) to account for different expression levels. A representative result of three independent experiments with high reproducibility is shown.
doi:10.1371/journal.pone.0099201.g004

HA mutant and clade 2.3.2.1 HA containing a D45N substitution. Recent studies characterizing escape mutants against anti-HA antibodies reported examples where HAs promote compensatory mutations in neuraminidase (NA) [37,38] or increase HA receptor-binding avidity [39] to acquire resistance against antibody-binding pressure. This may explain why the D45Y virus is resistant to OM-b and AY-2C2 mAbs. D45 is not likely to have a marked effect on the HA binding of these mAbs, but is likely to have an impact on the infectivity in the presence of OM-b or AY-2C2 mAbs. It is also possible that OM-b and AY-2C2 mAbs can bind with the D45Y HA on the cell surface, but not on the viral particle. In this instance, the NA protein may play a role in antibody binding; however, we have observed (ELISA and Western blot) that OM-b and AY-2C2 did not bind to a recombinant NA derived from the VN1194 virus [14].

There were differences in the MN activities of OM-b and AY-2C2 to the D43N/Y viruses selected by OM-b and those selected by AY-2C2. The HA titers of the D43N/Y viruses selected by AY-2C2 were low compared with those selected by OM-b at the same infectivity titers (data not shown). We consider that differences between the MN titers of escape mutants D43N/Y selected by OM-b and those selected by 2Y-2C2 may be due to changes in other genes such as NA [37,38].

The mAbs used in this study were specific to H5 HAs, and did not recognize other subtype viruses [14], with OM-b and AY-2C2 having a broad cross-reactivity among the H5N1 HAs in clades 1, 2.1.3.2, 2.3.4 [14], 2.2, 1.1, and 2.3.2.1. However, the OM-b and AY-2C2 coverage of human and avian H5N1 strains isolated from Africa in 2010–2013 was only 20.2% (data not shown). This is probably because the mAbs did not recognize several of the evolved clade 2.2.1 viruses [30,40]. Of note, we previously showed that OM-b and AY-2C2 mAbs were able to bind with A/turkey/Turkey/1/2005, which classified as an early clade 2.2.1 virus [14]. Also, OM-b mAb recognized additional 8 recombinant HA proteins originated from Asian H5N1 strains (clade 0, 1, 2.1.1, 2.2 and 2.3.4, all harboring 43D, 45D or N and 46G, unpublished

Table 6. Conservation of the epitopes among H5 Influenza viruses.

HA subtype	Virus host	OM-b and 2C2 epitope sequences/total number	Conservation rate (%)	
H5N1	Human	277/324	85.5	88.7
H5N1	Avian	2362/2652	89.1	
H5N2-N9	Avian	6/808	0.74	
H1N1pdm	Human, avian	0/1224	0	
HA subtype	**Virus host**	**1A1 epitope sequences/total number**	**Conservation rate (%)**	
H5N1	Human	212/327	64.8	60.6
H5N1	Avian	1615/2701	59.8	
H5N2-N9	Avian	725/769	94.3	
H1N1pdm	Human, avian	0/1224	0	

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data). Because the coverage of the Asian H5N1 strains was estimated to be as high as 96.5% (see Figure S2), we believe that OM-b and AY-2C2 mAbs are effective for the detection of H5N1 viruses in this geographical area. These observations are consistent with other studies of murine mAbs among these viruses [27,40]. To develop the methodology further, mAbs that recognize H5 HAs with D43N substitutions are needed to widen the range of detection for H5N1 strains.

Supporting Information

Figure S1 Structural comparison between H5 HA and chimeric HA. Loop structures of H5/1 chimeric HA (gray and blue, in the same order as Figure 3A) and VN1194 wild-type HA (orange) are superimposed. Left-hand diagram shows the structures of monomeric HAs. The main epitope sites of the C179 mAb are shown as blue (T318) and gray (V52) spheres. Orange spheres represent the wild-type HA. Right-hand diagram shows the structures of trimeric HAs.
(TIFF)

Figure S2 OM-b and AY-2C2 epitope conservation in human and avian H5N1 strains isolated in Asia during 2010–2013. (A) Distribution of the OM-b and AY-2C2 epitopes in Asia and the regional breakdown of non-conserved strains. HA sequences of the Asian H5N1 epidemic of 2010–2013 (Influenza Virus Resource database [27]) were multiple-aligned and analyzed for conservation of D43 and G46. (B) List of non-conserved strains.
(TIF)

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Author Contributions

Conceived and designed the experiments: MKI YTY. Performed the experiments: MKI HT. Analyzed the data: MKI HT. Contributed reagents/materials/analysis tools: KO KN SI TK. Wrote the paper: MKI HT. Helped to finalize manuscript: TK YTY. Provided useful suggestions and discussions: KO KT MA SI.

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