Journal of Clinical Microbiology

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J. Clin. Microbiol. **2011, 49(4):1542. DOI: 10.1128/JCM.01876-10.**
Published Ahead of Print 26 January 2011.

JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2011, p. 1542–1548 Vol. 49, No. 4
0095-1137/11/$12.00 doi:10.1128/JCM.01876-10

Utility of a Panviral Microarray for Detection of Swine Respiratory Viruses in Clinical Samples

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Received 14 September 2010/Returned for modification 16 November 2010/Accepted 14 January 2011

Several factors have recently converged, elevating the need for highly parallel diagnostic platforms that have the ability to detect many known, novel, and emerging pathogenic agents simultaneously. Panviral DNA microarrays represent the most robust approach for massively parallel viral surveillance and detection. The Virochip is a panviral DNA microarray that is capable of detecting all known viruses, as well as novel viruses related to known viral families, in a single assay and has been used to successfully identify known and novel viral agents in clinical human specimens. However, the usefulness and the sensitivity of the Virochip platform have not been tested on a set of clinical veterinary specimens with the high degree of genetic variance that is frequently observed with swine virus field isolates. In this report, we investigate the utility and sensitivity of the Virochip to positively detect swine viruses in both cell culture-derived samples and clinical swine samples. The Virochip successfully detected porcine reproductive and respiratory syndrome virus (PRRSV) in serum containing 6.10×10^2 viral copies per microliter and influenza A virus in lung lavage fluid containing 2.08×10^6 viral copies per microliter. The Virochip also successfully detected porcine circovirus type 2 (PCV2) in serum containing 2.50×10^8 viral copies per microliter and porcine respiratory coronavirus (PRCV) in turbinate tissue homogenate. Collectively, the data in this report demonstrate that the Virochip can successfully detect pathogenic viruses frequently found in swine in a variety of solid and liquid specimens, such as turbinate tissue homogenate and lung lavage fluid, as well as antemortem samples, such as serum.

Respiratory disease in pigs is the most important health concern for swine producers today. According to the 2006 NAHMS survey, respiratory disease was the greatest cause of mortality in swine, accounting for 53.7% of nursery deaths and 60.1% of deaths in grower/finisher pigs (22). The top viral pathogens affecting U.S. swine herds are porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV), swine influenza virus (SwIV), porcine circovirus type 2 (PCV2), and porcine respiratory coronavirus (PRCV).

PRRS disease was first recognized in 1987 in the United States (7). Since then, PRRS has become the number one swine disease problem in the United States, causing an estimated $570 million annual loss (13). The causative agent, PRRSV, is a single-stranded positive-sense RNA virus classified as a member of the genus *Arterivirus*, family *Arteriviridae*, and order *Nidovirales* (2). Due to its high mutation rate and degree of genetic variability, even among viruses within the same genotype, PRRSV has proven difficult to control and remains the foremost contributor to swine respiratory disease (12, 28). Accordingly, PRRS is an example of a continually emerging viral disease affecting swine and causing devastating effects on the industry.

SwIV is a member of the influenza A virus genus, which consists of enveloped viruses with a negative-sense RNA genome. Eight gene segments make up the RNA genome and encode the viral structural and nonstructural proteins. All influenza A viruses, including SwIV, undergo genetic variability or mutation as they circulate within host populations. This variance occurs both by random mutations within individual genes (genetic drift) and by reassortment (genetic shift). The relationship between swine and human influenza disease has long been recognized as important. Pigs are thought to be a "mixing vessel" or to act as a host capable of adaptation of avian influenza viruses to mammals, including humans. The bidirectional transmission of influenza viruses between pigs and humans has been documented. For example, isolation of a swine influenza virus from humans in 1974 confirmed that swine influenza viruses are zoonotic in nature (17). Further, H3 and H1N1 avian influenza virus subtypes have been isolated from pigs in Canada (6). During attendance at an Ohio county fair, both pigs and humans became clinically affected by an influenza-like illness caused by swine H1N1 virus A/SW/OH/511445/2007 (OH07) (23). The most recent example is the isolation of the novel H1N1 swine origin influenza A virus from two unrelated children in the San Diego, CA, area in April 2009, preceding the recognition of the 2009 flu pandemic (3, 4). The 2009 pandemic H1N1 virus contains a unique genome rearrangement consisting of six genes (the PB2, PB1, PA, hemagglutinin [HA], NP, and NS genes) that cluster with genes of viruses identified as triple-reassortment swine influenza viruses of the North American lineage and two genes (the M and NA genes) that are derived from Eurasian lineage swine viruses (4). Thus, monitoring influenza virus circulating in swine populations with a diagnostic assay that allows for detection of subtypes and genotypes novel to the swine popula-

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Published ahead of print on 26 January 2011.

tion is necessary, given the potential risk for human infection by transmission from clinically ill swine and the dramatic economic losses for the pork industry due to direct disease-related costs as well as indirect market losses.

PRCV, a member of the *Coronaviridae*, is a variant of transmissible gastroenteritis virus (TGEV) with altered tropism from the enteric tract to the respiratory tract. The main difference between PRCV and TGEV is a deletion in the S gene of PRCV (15, 25). Coronaviruses are enveloped, single-stranded RNA viruses that cause respiratory and enteric disease in animals, including the common cold and severe acute respiratory syndrome (SARS) in humans. PCV2 is a circular DNA virus that is ubiquitous in swine populations worldwide. Its economic importance lies in its association with the postweaning multisystemic wasting syndrome (PMWS), a condition initially observed with Canadian weaned pigs in 1991 (5). PCV2 is now regarded as an important swine pathogen.

Each of these viral pathogens causes substantial economic losses for the U.S. swine industry, which is in need of a detection and surveillance program that would quickly identify known pathogens and unknown agents as well. An estimated 75% of emerging diseases in humans arise from zoonotic sources (19). Major emerging infectious diseases in humans have originated in animal hosts, transferred either through direct transmission, such as with SARS and West Nile virus, or by mutations that may result in a "species jump," such as with human immunodeficiency virus (HIV), highly pathogenic avian influenza virus H5N1, and the most recent pandemic of novel H1N1 influenza virus. Domesticated animal populations throughout the world serve as an underappreciated and overlooked source of emerging zoonotic diseases of viral etiology.

PCR testing is rapid, highly sensitive, and the gold standard for detecting viruses in veterinary diagnostic settings. Unfortunately, most PCR tests target only one virus at a time, thus making these assays cumbersome when screening a clinical specimen for all viruses that have a PCR test available. Moreover, PCR cannot detect or identify new or novel viruses. Panviral DNA microarrays represent the most robust approach for massively parallel viral surveillance and detection. The Virochip is a panviral DNA microarray that is capable of detecting all known viruses, as well as novel viruses related to known viral families in a single assay (24). The Virochip has been used to indentify SARS (9, 24), xenotropic murine leukemia virus-related (a novel retrovirus) from patients with familial prostate cancer (21), and a novel clade of human rhinoviruses (8). The Virochip has also proven to be successful in a clinical veterinary setting by successfully identifying a novel coronavirus from a beluga whale held in an aquatic containment facility (11) and by identifying foot-and-mouth disease virus (FMDV) in ticks collected from a livestock market in Nairobi, Kenya (16). However, the usefulness and sensitivity of the Virochip platform have not been tested on a set of clinical veterinary specimens with the high degree of genetic variance that is frequently reported among swine viruses. In this report we investigate the usefulness and sensitivity of the Virochip to positively detect swine viruses in both cell culture samples and clinical swine samples. The swine viruses that we chose to investigate represent the most important endemic viruses with regard to their impact on health and economic losses to U.S. swine producers.

MATERIALS AND METHODS

Virus strains and clinical specimens. Type 2 PRRSV strains VR-2332 and MN184 were used in this study. Virus stocks were propagated on MARC-145 cells and were cultured and maintained in medium composed of minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 50 mg/liter of gentamicin (Gibco-Invitrogen) at 37°C, 5% CO_2. Clinical PRRSV samples were obtained from sera collected at 3 or 8 days postinfection (dpi) from three pigs infected with strain MN-184. Clinical SwIV samples were obtained from serum and a nasal swab placed in 2 ml MEM postcollection from a pig intratracheally inoculated with 2×10^5 50% tissue culture infective doses ($TCID_{50}$) of A/SW/OH/511445/2007 (OH07) rH1N1 as previously described (23). Clinical samples of the 2009 pandemic (H1N1) virus were obtained from nasal swabs placed in 2 ml MEM postcollection from pigs intratracheally inoculated with 2×10^5 $TCID_{50}$ of A/California/04/2009 (H1N1) as previously described (10). Clinical PCV2 samples were obtained from the serum and lung lavage fluid collected at 25 dpi from a gnotobiotic pig that had been inoculated with 1.5 ml PCV2-positive sera intramuscularly, 1.5 ml intranasally, and 1.5 ml intraperitoneally. The clinical PRCV sample was nasal turbinate isolated from a pig inoculated intranasally with 4 ml (2 ml/nostril) of cell culture medium containing 10^6 median cell culture infectious doses ($CCID_{50}$)/ml of PRCV. The nasal turbinate specimen was weighed and ground in phosphate-buffered saline (PBS) to make a 10% weight-to-volume suspension (1).

RNA extraction and specimen processing. Viral RNA was purified from the different samples with the MagMAX-96 viral RNA isolation kit (Ambion), according to the manufacturer's instructions. Once RNA was isolated, quantification of total RNA was achieved by using a Nanodrop spectrophotometer (Eppendorf).

Panviral DNA microarray analysis. For each sample, two separate random amplification reactions were generated using 50 nanograms of viral RNA following the round A/B/C random amplification protocol (protocol S1 of Wang et al. [24]). Postamplification, one reaction was subsequently labeled and hybridized to the Virochip as previously described (protocol S1 of Wang et al. [24]). The second reaction was then used for quantitative reverse transcription (qRT)-PCR analysis to quantitate viral copies present in each sample after the random amplification process. Microarrays were scanned using the Axon 4000B scanner and GenePix software (Molecular Devices). Hybridization signatures were interpreted by an individual (D. Wang) blinded from sample information and qRT-PCR results. E-predict, a computational tool developed for analyzing the Virochip array hybridization signature, was used to determine all Virochip results, with a *P* value cutoff positivity of 0.05, as described elsewhere (20).

qRT-PCR. PRRSV was quantitated using a TaqMan-based real-time RT-PCR. DNA (569 bp) coding for nucleotides 14762 to 15330 of strain Ingelvac PRRS MLV, amplified with forward primer p6/385 (5′-TAACCACGCATTTG TCGTCC) and P7/3341R (5′-GTCAATCAGTGCCATTCACC), was cloned into pGEM-T (Promega Corporation), and *in vitro* transcription was performed to synthesize viral RNA using RiboMAX large-scale RNA production system T7 (Promega Corporation). The RNA copy number was calculated using Avogadro's number (6.022×10^{23} copies/mole) divided by the molecular weight of one copy of the transcript (223,898 g/mol) to derive the number of copies per gram of *in vitro*-derived transcript (2.69×10^{18}). A standard curve was then derived using serial 10-fold dilutions of the viral transcript (10^5 to 10^{10}). RNA from each serum sample was extracted as described above and then amplified and detected in a TaqMan-based assay run in triplicate. The mean RNA copy number was calculated from a concurrently run standard curve. The TaqMan assay utilized 8 μl of extracted RNA and the Qiagen one-step RT-PCR kit supplemented with a final concentration of 3 mM $MgCl_2$, 0.2 μg/μl bovine serum albumin, RNaseOUT recombinant RNase inhibitor (Invitrogen), 0.4 pmol/μl forward primer PRRS US-F (5′-TCAGCTGTGCCAGATGCTGG), 0.4 pmol/μl reverse primer PRRS US-R (5′-AAATGCGGCTTCTCCGGGTTTT), and 0.1 μM probe (5′-6-carboxyfluorescein-TCCCGGTCCCTTGCCTCTGGA-6-carboxytetramethylrhodamine) (25-μl final volume/reaction mixture). Cycling conditions were as follows: 1 cycle of 30 min at 50°C, 1 cycle of 15 min at 95°C, and 45 cycles of 94°C for 15 s and 60°C for 1 min. Clinical (OH07) SwIV was quantitated using a modified TaqMan-based qRT-PCR for the conserved matrix (M) gene. For generation of a standard curve, the entire matrix gene was RT-PCR amplified with primers to the conserved 12 and 13 bp present on the 5′ and 3′ ends of the gene of an avian influenza virus isolate (18). Ten-fold dilutions of a 1-pg/μl solution were used to construct a standard curve. The modified TaqMan procedure utilized the TaqMan one-step RT-PCR master mix reagent

TABLE 1. Number of SNPs present in viral genome sequence compared to PRRSV-specific oligonucleotide probe sequence by the panviral microarray[a]

PRRSV strain	Oligonucleotide probe ID	Gene	No. of SNPs
VR-2332	9630807_309/9630807_309_rc	ORF1	2
	9630807_454/9630807_454_rc	ORF1	2
	9630807_531/9630807_531_rc	ORF3/4	0
	9630807_325/9630807_325_rc	ORF1	0
	9630807_346/9630807_346_rc	ORF1	1
MN184	9630807_309/9630807_309_rc	ORF1	1
	9630807_454/9630807_454_rc	ORF1	8
	9630807_531/9630807_531_rc	ORF3/4	7
	9630807_325/9630807_325_rc	ORF1	11
	9630807_346/9630807_346_rc	ORF1	9

[a] The genomic sequences of PRRSV strains VR-2332 (GenBank accession no. U87392) and MN-184 (EF488739) were used for comparison.

kit (Applied Biosystems), 0.5 pmol/μl forward primer, 0.5 pmol/μl reverse primer, and 0.1 μl probe. Clinical pandemic (H1N1) 2009 virus was quantitated using an established qRT-PCR method used to detect the pandemic influenza A virus (H1N1) 2009 matrix gene in swine samples (10). An established qRT-PCR procedure was used to derive the quantity of PCV2 DNA in the swine samples (14). Currently there is no qRT-PCR assay for PRCV in use at the National Animal Disease Center.

RESULTS

Detection of PRRSV from cell culture and spiked serum samples by the Virochip. Due to the high degree of genetic variability, even among viruses within the same genotype, we initially examined the sequence variability between the PRRSV-specific oligonucleotide probe sequences present on the Virochip and the corresponding viral sequences for VR-2332 and MN-184. This comparison revealed a low degree of sequence variation for one PRRSV strain and a high degree for the other strain and their corresponding probe sequences. Specifically, the number of single-nucleotide polymorphisms (SNPs) identified ranged between 0 and 11 (Table 1). Two out of five probe sequence sets present on the Virochip were identical to the corresponding viral sequence for VR-2332, and none of the probe sequences were identical to that of strain MN-184 (Table 1).

To begin testing the usefulness of the Virochip to positively detect PRRSV, cDNA generated from RNA isolated from VR-2332-infected tissue culture cells was randomly amplified, labeled, and hybridized to the Virochip. qRT-PCR analysis was performed to quantitate the number of viral copies per microliter present in each sample after the random amplification process (Table 2). The hybridization data positively identified the presence of PRRSV in this sample, indicating that the Virochip is capable of successfully identifying the virus in cell culture (Table 2).

We then sought to determine if the Virochip could positively detect PRRSV in serum and, if successful, discern the lowest PRRSV titer that could be successfully detected by the Virochip. Strain MN-184 was added to uninfected serum to achieve a viral titer of 10^6 $TCID_{50}$ per ml. Tenfold dilutions of this spiked serum, using uninfected serum as diluent, were then generated, and qRT-PCR analysis was performed to quantitate the number of viral copies per microliter present in each sample after the random amplification process (Table 2). PRRSV was positively detected by qRT-PCR in the undiluted spiked serum and in the $10^{-1}$and 10^{-2} dilutions, while PRRSV failed to be detected in the 10^{-3} and 10^{-4} dilutions (Table 2). Randomly amplified cDNA generated from RNA isolated from each spiked serum sample (undiluted 10^6 per ml spiked serum and 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions) was labeled and hybridized to the Virochip. Hybridization results demonstrated that PRRSV was positively identified in the undiluted PRRSV-spiked serum sample and in the 10^{-1} dilution (Table 2). However, the Virochip failed to identify PRRSV in the 10^{-2} dilution of the PRRSV-spiked serum as well as in the 10^{-3} and 10^{-4} dilutions, in which PRRSV was also not detected by qRT-PCR (Table 2). When differences in the hybridization signal intensity between cell culture samples and serum samples were evaluated, we found no difference between the undiluted 10^6-per-ml spiked serum sample and the cell culture sample (Fig. 1). However, a large decrease in the hybridization signal intensity was detected between the undiluted 10^6-per-ml spiked serum sample and the 10^{-1} dilution (Fig. 1). The relative insensitivity of strain MN-184 added to uninfected serum by the Virochip, compared to qRT-PCR, is likely due to the number of differences in the nucleotide sequence seen between the viral and Virochip probe sequences. The positive identification of PRRSV in the undiluted PRRSV-spiked serum by the Virochip demonstrates that the Virochip can detect PRRSV from serum samples with a minimum titer of 10^5 $TCID_{50}$ per ml. Additionally, these results show that the Virochip is capable of successfully identifying a PRRSV strain containing a high degree of sequence variability between the viral sequence and the corresponding PRRSV-specific oligonucleotide probe sequences present on the Virochip (Table 2).

Virus detection in clinical swine samples by the Virochip. To assess the performance of the Virochip in a clinical veterinary setting, we first examined serum samples collected from PRRSV-infected pigs. We specifically chose to evaluate serum

TABLE 2. Detection of PRRSV by Virochip[a]

Sample no.	Virus	Sample information	Viral copies per μl postamplification	Array result
1	VR-2332	RNA isolated from infected tissue culture cells	2.31×10^8	PRRSV
2	MN-184	Spiked serum, 10^6 $TCID_{50}$/ml	1.87×10^4	PRRSV
3	MN-184	Spiked serum, 10^5 $TCID_{50}$/ml	2.55×10^3	PRRSV
4	MN-184	Spiked serum, 10^4 $TCID_{50}$/ml	8.31×10^2	ND
5	MN-184	Spiked serum, 10^3 $TCID_{50}$/ml	ND	ND
6	MN-184	Spiked serum, 10^2 $TCID_{50}$/ml	ND	ND

[a] ND, not detected.

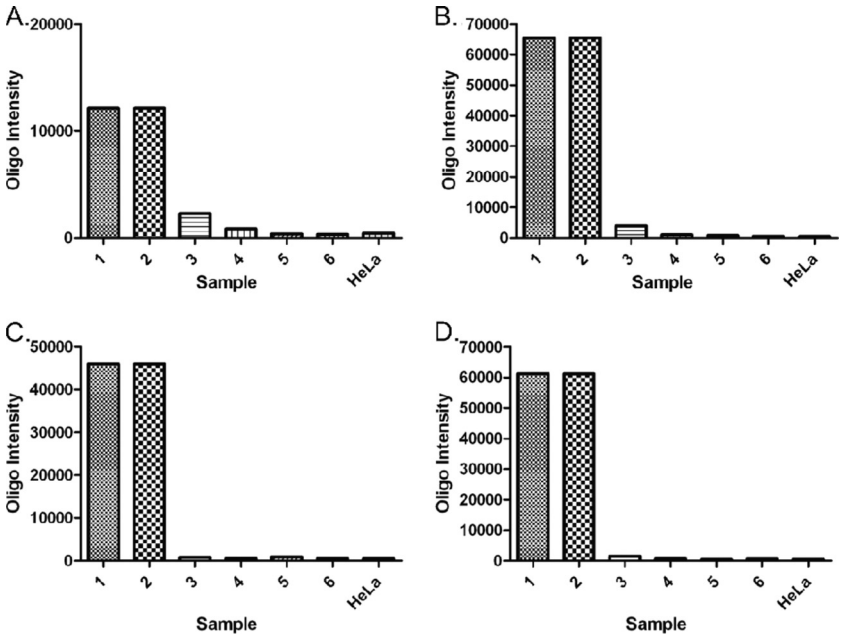

FIG. 1. Hybridization intensity plots of PRRSV-specific oligonucleotide probes from the Virochip. Oligonucleotide probes 9630807_454 (A), 9630807_454_rc (B), 9630807_325_rc (C), and 9630807_346_rc (D) contain a 2-, 2-, 0-, and 1-bp mismatch, respectively, between the probe sequence and that of strain VR-2332. The respective probes each contain an 8-, 8-, 11-, and 10-bp mismatch with strain MN184 (Table 1). Oligonucleotide intensities are plotted along the *y* axis. Samples 1 (VR-2332) and 2 to 6 (MN184 dilutions) (Table 2), along with a HeLa control, are plotted along the *x* axis.

samples because serum is easy to collect, in that it does not require extensive training and can be collected antemortem, allowing multiple samples to be drawn from a single animal, making serum an excellent choice for use in disease surveillance programs. cDNA generated from RNA isolated from sera collected from five pigs infected with PRRSV strain MN-184 was randomly amplified, labeled, and hybridized to the Virochip. qRT-PCR analysis showed that the number of viral copies per microliter present in each of these samples after the random amplification process ranged from 6.10×10^2 to 3.47×10^6 (Table 3). The hybridization data positively identified the presence of PRRSV in all six clinical serum samples (Table 3). These results directly demonstrate that the Virochip is capable of successfully identifying PRRSV in clinical serum samples even when they are collected from swine infected with a PRRSV strain containing a high degree of sequence variability, such as MN-184.

Subsequently, we examined the ability of the Virochip to positively detect influenza A virus in clinical swine samples collected from pigs experimentally infected with either the pandemic (H1N1) 2009 strain or the OH07 swH1N1 strain. We first compared the sequence variabilities between the influenza A virus-specific oligonucleotide probe sequences present on the Virochip and the corresponding viral sequence for the (H1N1) 2009 strain and the OH07 swH1N1 strain. This comparison revealed a high degree of sequence variation for both

TABLE 3. Detection of viruses in clinical swine samples by Virochip

Sample no.	Virus	Sample	Viral copies per μl postamplification[a]	Array result
7	PRRSV (MN-184)	Serum	3.09×10^4	PRRSV
8	PRRSV (MN-184)	Serum	1.89×10^5	PRRSV
9	PRRSV (MN-184)	Serum	4.53×10^5	PRRSV
10	PRRSV (MN-184)	Serum	3.47×10^6	PRRSV
11	PRRSV (MN-184)	Serum	7.82×10^2	PRRSV
12	PRRSV (MN-184)	Serum	6.10×10^2	PRRSV
13	Influenza A (pandemic H1N1 2009)	Nasal swab	3.10×10^6	Negative
14	Influenza A (pandemic H1N1 2009)	Nasal swab	4.25×10^2	Negative
15	Influenza A (pandemic H1N1 2009)	Nasal swab	7.80×10^4	Negative
16	SwIV (OH07)	Nasal swab	1.51×10^1	Negative
17	SwIV (OH07)	Lung lavage fluid	2.08×10^6	Influenza A virus
18	PCV2	Serum	2.50×10^8	Porcine circovirus
19	PCV2	Lung lavage fluid	2.94×10^8	Porcine circovirus
20	PRCV	Turbinate tissue homogenate	NA	Coronavirus

[a] NA, not available.

TABLE 4. Number of SNPs present in viral genome sequence compared to influenza-specific oligonucleotide probe sequence[a]

Influenza virus	Oligonucleotide probe ID	Gene	No. of SNPs
OH07 swH1N1	8486129_2/8486129_2_rc	NP	10
	8486129_20/8486129_20_rc	NP	9
	8486129_38/8486129_38_rc	NP	15 + 1-base deletion
	8486129_56/8486129_56_rc	NP	5
	8486129_29/8486129_29_rc	NP	7
	8486129_59	NP	2
	8486125_68/8486125_68_rc	HA	10
	8486125_53/8486125_53_rc	HA	10
	8486125_61/8486125_61_rc	HA	14 + 3-base insertion
2009 H1N1	8486129_2/8486129_2_rc	NP	10
	8486129_20/8486129_20_rc	NP	7
	8486129_38/8486129_38_rc	NP	12 + 1 base-deletion
	8486129_56/8486129_56_rc	NP	6
	8486129_29/8486129_29_rc	NP	8
	8486129_59	NP	3
	8486125_68/8486125_68_rc	HA	10
	8486125_53/8486125_53_rc	HA	8
	8486125_61/8486125_61_rc	HA	12 + 3-base insertion

[a] The genomic sequences of influenza strains A/swine/OH/511445/2007(H1N1) [EU604694(NP) and EU604689(HA)] and A/CA/04/2009 [FJ969512(NP) and GQ117044(HA)] were used for comparison.

strains and their corresponding probe sequences (Table 4). The clinical samples we focused on evaluating were nasal swabs because the virus is restricted to the respiratory tract; as such, swabs thus serve as an excellent choice for use in antemortem SwIV disease surveillance programs. First, randomly amplified cDNA generated from RNA isolated from nasal swabs collected from pigs infected with the pandemic (H1N1) 2009 strain were labeled and hybridized to the Virochip. Additionally, qRT-PCR analysis was performed to quantitate the number of viral copies per microliter present in each sample after the random amplification process (Table 3). The hybridization data failed to identify the presence of influenza A virus in all three samples (Table 3). Next, nasal swab and lung lavage fluid samples were collected from a pig experimentally infected with OH07 swH1N1 influenza virus. Randomly amplified cDNA generated from RNA isolated from these samples was labeled and hybridized to the Virochip. qRT-PCR analysis was used to determine that the nasal swab sample contained 1.51×10^1 and the lung lavage sample contained 2.08×10^6 viral copies per microliter after the random amplification process (Table 3). The hybridization data failed to identify the presence of influenza A virus in the nasal swab sample; however, influenza A virus was positively identified in the lung lavage fluid sample (Table 3). The positive identification of influenza A virus in the lung lavage fluid sample indicates that the Virochip is capable of successfully identifying influenza A virus in clinical swine samples of sufficient viral RNA quantity.

Next we evaluated the ability of the Virochip to positively detect two other main swine respiratory pathogens, PCV2 and PRCV, in clinical swine samples collected from pigs experimentally infected with each of these viruses. RNA isolated from serum and lung lavage samples collected from a pig experimentally infected with PCV2 was used to generate randomly amplified cDNA, which was then labeled and hybridized to the Virochip. Using qRT-PCR, we determined that the serum and lung lavage fluid samples contained 2.50×10^8 and 2.94×10^8 viral copies per microliter, respectively, after the random amplification process (Table 3). The hybridization data positively identified porcine circovirus in both the serum and lung lavage fluid clinical samples (Table 3). To test the ability of the Virochip to positively identify PRCV, RNA isolated from turbinate tissue homogenate collected from a pig infected with porcine coronavirus was used to generate randomly amplified cDNA, labeled, and hybridized to the Virochip. The hybridization data positively detected coronavirus in the turbinate tissue homogenate (Table 3). Taken together, these data demonstrate that the Virochip can positively identify PCV2 and PRCV in clinical swine samples.

DISCUSSION

The domesticated animal populations within the United States serve as an undersurveilled source for emerging zoonotic diseases of viral etiology. Thus, there is a substantial need for surveillance programs coupled with next-generation detection methods for both human and animal health preparedness for future outbreaks. Additionally, domesticated animal populations within the United States serve as an invaluable resource for investigative studies specifically addressing viral discovery and viral evolution. Panviral DNA microarrays represent one approach for massively parallel viral surveillance and discovery. One advantage of using the Virochip platform is its unambiguous and unbiased ability to potentially detect many viral strains in parallel. The Virochip has proven to be extremely successful in both human clinical settings and veterinary clinical settings (8, 9, 11, 16, 21, 24). A recent example includes the successful identification of a novel coronavirus from a beluga whale held in an aquatic containment facility by the Virochip (11). The ability of the Virochip to comprehensively detect known and novel viruses in a single assay makes it an appealing choice for surveillance, detection, and discovery programs. However, several considerations need to be addressed before this technology is utilized in a clinical veterinary setting. First, setting up the technology can entail significant initial startup costs, which include the cost of the viral oligonucleotides. Second, the turnaround time for the assay is around 24 h. Third, and more importantly, the usefulness and the sensitivity of the Virochip platform need to be tested on a diverse set of clinical veterinary specimens from domestic livestock. In this report we investigate the usefulness and sensitivity of the Virochip to positively detect swine viruses in both cell culture samples and clinical swine samples. The swine viruses that we chose to investigate have the most important impact on both health and the economic loss to U.S. swine producers.

Here we have demonstrated that the Virochip can readily detect PRRSV in both cell culture and spiked serum samples. A large decrease in the hybridization signal intensity occurred when the viral titer of the spiked serum was diluted from 10^6 per ml to 10^5 per ml. These data directly correlate sample viral titers with successful detection by the Virochip and highlight the importance of sensitivity when utilizing the Virochip platform in a clinical veterinary setting. Combining this result with the SNPs identified between the Virochip probe sequences and strain MN-184 illustrates how the sensitivity of the Virochip platform could be improved by modifying the probe design.

For example, designing and adding probes specifically targeting the nucleocapsid protein (ORF7) of the PRRSV genome, which is known to be present in greater quantities during infection, especially in easily acquired clinical samples, such as serum (26, 27), would greatly increase the sensitivity of the Virochip for the detection of PRRSV in swine samples. Additionally, designing and adding probes targeting the 5′ and 3′ untranslated regions, as these two regions have been shown to have the highest degree of conservation and are expressed in abundances equal to those seen with ORF7, would increase the sensitivity of the Virochip for the detection of PRRSV. When we assessed the performance of the Virochip in a clinical veterinary setting, the Virochip positively identified PRRSV in all six serum samples collected from PRRSV-infected pigs. However, all six serum samples contained a relatively high number of viral copies. These data demonstrate that the Virochip, without any improvements with regard to sensitivity, can successfully identify PRRSV in antemortem clinical samples containing a relatively high viral titer. Taken together, the results from the spiked serum and clinical samples suggest that improvements in the sensitivity of the Virochip, such as those gained by probe modifications, would make the Virochip extremely useful as a viral discovery platform in clinical veterinary settings.

To examine the ability of the Virochip to positively detect influenza A virus in clinical swine samples, we utilized randomly amplified cDNA generated from RNA isolated from nasal swab and lung lavage fluid samples taken from a pig infected with OH07 swH1N1 influenza virus (23) and nasal swabs taken from pigs infected with the pandemic (H1N1) 2009 strain. The Virochip successfully identified influenza A virus in lung lavage fluid from the pig infected with OH07 swH1N1 but failed to detect influenza A virus in the nasal swab. qRT-PCR demonstrated that the nasal swab sample contained 1.51×10^1 viral copies per microliter after the random amplification process, which is low and approaches the limit of detection for the assay. Given that the Virochip successfully identified influenza A virus in the lung lavage fluid sample, the failed detection of influenza A virus in the nasal swab is likely due to the low number of viral copies present in this nasal swab. The positive identification of influenza A virus in the lung lavage fluid sample demonstrates that the Virochip can positively detect swine influenza viruses in clinical samples, including zoonotic isolates for which pig-to-human transmission has been documented, such as OH07 swH1N1. However, the Virochip failed to identify the presence of influenza A virus in all three nasal swab samples taken from pigs infected with the pandemic (H1N1) 2009 strain. Both the HA and NA genes of the pandemic (H1N1) 2009 strain are phylogenetically related more closely to the North American lineage and Eurasian lineage of swine viruses (4), respectively, than to human influenza A viruses for which the probes of the Virochip are designed. This is reflected in the number of SNPs present in the 2009 H1N1 genome sequence compared to that in the influenza virus-specific oligonucleotide probe sequence (Table 4). Designing and adding probes specifically targeting the HA and NA genes of the North American lineage and Eurasian lineage of swine viruses would likely increase the sensitivity of the Virochip for the detection of influenza A virus in clinical swine samples. Similarly, the addition of probes specifically targeting the conserved regions within the NP or M gene would likely increase the sensitivity as well.

To further assess the performance of the Virochip in the clinical veterinary setting, randomly amplified cDNA generated from RNA isolated from sera and lung lavage fluid taken from pigs infected with PCV2 was labeled and hybridized to the Virochip. The Virochip positively identified the presence of porcine circovirus in both clinical specimens. When we examined the ability of the Virochip to positively detect coronavirus in a clinical swine sample, we utilized randomly amplified cDNA generated from RNA isolated from turbinate tissue homogenate taken from a coronavirus-infected pig. The Virochip successfully identified coronavirus in this solid clinical swine sample and demonstrates that the Virochip can successfully detect viruses for which there are no RT-PCR assays available for clinical veterinary samples.

Together, the data in this report demonstrate that the Virochip can successfully detect pathogenic viruses frequently found in swine in a variety of solid specimens, such as turbinate tissue homogenate, and liquid specimens, such as serum and lung lavage fluid. This is the first documented report demonstrating the successful use of the Virochip platform to detect major endemic swine viruses with regard to their impact on health and economic losses to U.S. swine producers, in a number of different clinical samples, including samples collected antemortem. The ability of the Virochip to positively detect viruses with a high degree of genetic variance, as is found in the clinical swine specimens tested here, is a benefit that may outweigh concerns regarding costs and turnaround time. Furthermore, the advantages in the technical effort, cost, and turnaround time involved in using the Virochip as a viral discovery platform far exceed those of next-generation sequencing platforms. Nonetheless, improvements in the sensitivity of the Virochip are needed for routine diagnostic needs. By adding a small number of probes specifically targeting the HA and NA genes of the swine lineage influenza A viruses, the Virochip platform can successfully be used to monitor influenza virus subtypes and genotypes circulating in swine populations or zoonotic viruses emerging in the human population. These improvements combined with other probe modifications that lead to improved sensitivity will make the Virochip an unparalleled viral discovery tool for the clinical veterinary setting.

ACKNOWLEDGMENTS

We are deeply indebted to David Wang and members of the Wang laboratory for technical assistance and advice. Additionally, we are greatly appreciative of Guang Wu, Sarah Pohl, Ann Vorwald, and Sarah Shore for excellent technical support.

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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