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A line immunoassay utilizing recombinant nucleocapsid proteins for detection of antibodies to human coronaviruses

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Abstract

Most coronaviruses infecting humans cause mild diseases, whereas severe acute respiratory syndrome (SARS)-associated coronavirus is an extremely dangerous pathogen. Here, we report the development of a serologic assay for detection of antibodies to human coronaviruses (HCoVs) based on recombinant nucleocapsid (N) proteins of all known pathogenic strains (229E, NL63, OC43, HKU1, SARS). The novel immunoassay is highly useful for epidemiologic surveys, where use of nucleic acid diagnostics often is limited. Purified recombinant antigens were immobilized on nitrocellulose membranes and applied in a line immunoassay, which allows rapid detection of antibodies to 5 different HCoVs in a single experiment. For assay evaluation, serum samples from persons infected with 229E or OC43 (acute/convalescent), recovered SARS patients and healthy donors were analyzed. Screening for nucleocapsid (N)-specific immunoglobulin G (IgG) in convalescent sera reached 100% sensitivity. With this new technique, we found that recently identified NL63 and HKU1 contribute significantly to the overall spectrum of coronavirus infections. Possibly, cross-reactive antibody responses were observed using 229E and OC43 serum pairs. However, the potential of this assay could clearly be demonstrated employing SARS-positive serum samples, where nonspecific binding to nucleocapsids of other HCoVs was not observed. This coronavirus strain-specific line immunoassay represents a powerful tool for serologic diagnostics.

Keywords: Human coronavirus; Nucleocapsid; Line immunoassay; SARS; Seroprevalence; Cross-reaction

1. Introduction

Coronaviruses (family Coronaviridae, order Nidovirales) are large (120–160 nm), enveloped, roughly spherical particles. They possess a nonsegmented single-stranded RNA genome of up to 31 kb in positive orientation. Based on serologic cross-reactivities and sequence analysis, coronaviruses are classified in 3 distinct groups (Eickmann et al., 2003; Gorbalenya et al., 2004; Kim et al., 2006; Weiss and Navas-Martin, 2005). Coronaviruses are known to infect many domestic animals as well as humans, causing acute and chronic diseases of the respiratory, enteric, and central nervous system (Weiss and Navas-Martin, 2005). Human coronaviruses (HCoVs) 229E and OC43 have 1st been described in the 1960s (Hamre et al., 1967; McIntosh et al., 1967) and are thought to be responsible for 10% to 30% of all common colds (Larson et al., 1980; Myint, 2006). The new infectious disease severe acute respiratory syndrome (SARS) emerged in November 2002 in Guangdong Province, People's Republic of China. Within a few months, SARS had spread to 26 countries, infecting more than 8000 people of whom almost 10% died (Poutanen and Low, 2004). The etiologic agent was recognized to be a novel virus, termed *SARS-CoV* (Drosten et al., 2003), which is related to, but distinct from other, known coronaviruses. Recent findings suggest a close relationship between SARS-CoV and group II coronaviruses (Kim et al., 2006). In 2004, van der Hoek et al. (2004) reported the isolation of a new group I CoV from a 7-month-old child with bronchiolitis,

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named HCoV-NL63. Independently described viruses HCoV-NL (Fouchier et al., 2004) and HCoV-NH (Esper et al., 2005b) are closely related to NL63 and likely represent strains of the same species of virus (Kahn, 2006; van der Hoek and Berkhout, 2005). HCoV-NL63 has been linked to more severe ailments, such as morbus croup (van der Hoek et al., 2005), and a systemic vasculitis in young children, Kawasaki disease, although association with the latter is discussed controversially (Esper et al., 2005a; Shimizu et al., 2005). NL63 infections have been reported throughout the world (Arden et al., 2005; Bastien et al., 2005; Chiu et al., 2005; Garbino et al., 2006), indicating that this HCoV can be regarded as a new major etiologic agent of respiratory disorders. HCoV-HKU1 was 1st isolated 2005 in Hong Kong from a 71-year-old man suffering from pneumonia and was classified as group II coronavirus (Woo et al., 2005a). So far, this strain has been detected in Asia, Europe, Unites States, and Australia in connection with respiratory and enteric tract illness (Esper et al., 2006; Garbino et al., 2006; Lau et al., 2006; Sloots et al., 2006; Vabret et al., 2006).

Coronavirus diagnostics are dominated by nucleic acid technologies (NATs). Because reconvalescence leads to a loss of viremia, NATs are not applicable for coronavirus screening in epidemiologic surveys outside acute pandemics (e.g., SARS). Serology so far is rarely used and not yet developed to distinguish between pathogenic types of HCoVs.

As candidate antigens for immunoassays detecting anti-coronavirus antibodies, structural proteins are of special interest. The RNA binding nucleocapsid (N) protein exhibits high immunogenicity and is abundantly produced during infection (Timani et al., 2004). Several methods using nucleocapsids as diagnostic antigens have been designed with special emphasis on SARS N (Chen et al., 2005; Guan et al., 2004; Liang et al., 2005; Tan et al., 2004). Here, we describe the establishment of a line immunoassay for rapid and simultaneous detection of antibodies directed against the 5 known HCoVs (229E, NL63, OC43, HKU1, and SARS) in human sera based on recombinant viral N proteins. We cloned and expressed the corresponding viral genes in *Escherichia coli*. Purified proteins were subsequently employed in a line immunoassay and tested for suitability as diagnostic antigens.

2. Materials and methods

2.1. Viral strains and viral RNA

HCoVs 229E (ATCC VR-740) and OC43 (ATCC VR-759) were obtained from the American Type Culture Collection (Manassas, VA) and propagated on MRC-5 cells. RNA was isolated from cell culture supernatant using the SV Total RNA Isolation System (Promega, Mannheim, Germany). HCoV-NL63-RNA was kindly provided by L. van der Hoek, Amsterdam, Netherlands. HKU1-RNA (strain Caen) was received from A. Vabret, Caen, France, and SARS-CoV-RNA (strain FRA) was prepared and sent to our laboratory by M. Eickmann, Marburg, Germany.

2.2. Reverse transcriptase polymerase chain reaction, polymerase chain reaction and cloning of coronaviral nucleocapsid genes

cDNA was synthesized by M-MLV reverse transcriptase (Promega) using gene-specific reverse primers derived from full genome sequences (see Table 1 for GenBank accession numbers). Subsequently, cDNA was amplified utilizing PfuTurbo® hotstart DNA polymerase (Stratagene, Heidelberg, Germany) with primer pairs displayed in Table 1. Cloning sites overlapping the start codon and cloning sites downstream of the stop codon were introduced as indicated. All oligonucleotides were synthesized by Metabion, Martinsried, Germany.

The resulting polymerase chain reaction (PCR) products were cloned into expression vector pCS04 (Lindner, unpublished), a derivative of pET22b (Novagen, Schwalbach,

Table 1
Accession numbers and oligonucleotides used for reverse transcription and amplification of cDNA

HCoV	Accession number, position	Primer (5′→3′)	Cloning site
229E	DQ243939[a]	GAACGAA**CATATG**GCTACAGTCAAATGGG	*Nde*I
(VR-740)		GT**GGATCC**TTTAGTTTAC	*Bam*HI
OC43	AY585228[b]	AAATTTTA**CATATG**TCTTTTACTCCTGG	*Nde*I
(VR-759)	29079–30425[c]	GGT**GAATTC**TCTTATATTTCT	*Eco*RI
NL63	AY567487[b]	TAAACTAAAC**CATATG**GCTAGTG	*Nde*I
	26133–27266[c]	TG**GAATTC**CAAAACAATTTAATGC	*Eco*RI
SARS (FRA)	AY310120[b]	ACAAATT**CATATG**TCTGATAATGG	*Nde*I
	28120–29388[c]	GG**GGATCC**TGAGTGTTTATGCC	*Bam*HI
HKU1 (Caen)	DQ778921[a]	ATCTA**CCCGCTTAGT**ATGTCTTATAC	*Fau*I
		ATTA**GAATTC**ATTCTCAATTAAG	*Eco*RI

Boldface letters in primer sequences indicate restriction endonuclease cleavage sites.

[a] Accession numbers for nucleocapsid genes.

[b] Accession numbers for full genome sequences.

[c] Nucleotide positions of corresponding nucleocapsid genes.