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# Development and characterization of polyclonal peptide antibodies for the detection of Yellow fever virus proteins

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There is still a considerable need for development of new tools and methods detecting specific viral proteins for the diagnosis and pathogenesis study of the Yellow fever virus (YFV). This study aimed to develop and characterize polyclonal peptide antisera for detection of YFV-C and -NS1 proteins. The antisera were used further to investigate NS1 protein expression during YFV infection in mammalian cells.

YFV target proteins were detected by all antisera in western blot and immunofluorescence assays. No cross-reactivity was observed with Dengue virus, West Nile virus, Tick-borne encephalitis virus and Japanese encephalitis virus. Nuclear localization of the YFV-C protein was demonstrated for the first time. Experiments investigating NS1 expression suggested a potential use of the YFV-NS1 antisera for development of diagnostic approaches targeting the secreted form of the NS1 protein.

The antisera described in this study offer new possibilities for use in YFV research and for the development of novel diagnostic tests.



## 1. Introduction

Yellow fever (YF) is a severe viral infectious disease caused by the Yellow fever virus (YFV) which is transmitted by mosquitoes in tropical regions of Africa and South America (WHO, 2010). Despite being vaccine preventable, YF still has a high impact upon morbidity and mortality amongst populations in endemic regions with an estimated 130,000 severe cases and 78,000 deaths per year in Africa alone (Garske et al., 2014). Maintaining adequate vaccine coverage remains challenging mainly due to logistic and economic reasons, and also the development of specific YF therapy has not been successful thus far.

The YFV belongs to the family *Flaviviridae*, together with other important human pathogenic viruses including Dengue virus (DENV), West Nile virus (WNV), Tick-borne encephalitis virus (TBEV) and Japanese encephalitis virus (JEV). Flaviviruses are small enveloped viruses composed of three structural and seven non-structural proteins (Chambers et al., 1990; Mukhopadhyay et al., 2005). The structural features of several flaviviral proteins have been determined and although such data is not yet available for YFV, there is assumed homology in its structural and antigenic properties with other flaviviruses (Luca et al., 2012; Nayak et al., 2009; Nybakken et al., 2006; Kanai et al., 2006; Elahi et al., 2013; Kiermayr et al., 2009; Ma et al., 2004; Akey et al., 2014; Edeling et al., 2014).

The capsid (C) protein forms the viral capsid and interacts with the RNA and the surrounding membrane (Ma et al., 2004). In addition, a location of the C protein in the nucleus of infected cells and interaction with different cellular proteins has been documented for several flaviviruses, implying a relevant role in viral replication and the virus-host interplay (Wang et al., 2002; Mori et al., 2005; Balinsky et al., 2013; Bulich and Aaskov, 1992; Westaway et al., 1997; Bhuvanakantham et al., 2009; Colpitts et al., 2011). Analogous localization and interaction has not yet been proven for YFV.

The highly conserved non-structural protein 1 (NS1) is an essential part of the viral replication complex and interferes with the immune response. It appears in a membrane-associated form on the surface of infected cells and in a secreted form which is released

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**Table 1**
Sequence and position of peptides used for the production of YFV specific antisera.

| Peptide | Position in the YFV protein | Target protein | Peptide sequence | Antiserum | Species |
|---|---|---|---|---|---|
| C | 28–42 | YFV-C | C-KQK TKQ IGN RPG PSR | Anti-YFV-C | Rabbit |
| NS1-1 | 91–105 | YFV-NS1 | QDP KNV YQR GTH PFS-C | Anti-YFV-NS1-1 | Rabbit |
| NS1-2 | 128–143 | YFV-NS1 | RKN GSF IID GKS RKE C | Anti-YFV-NS1-2 | Guinea pig |
| NS1-3 | 188–202 | YFV-NS1 | VNG KKS AHG SPT FWM-C | Anti-YFV-NS1-3 | Guinea pig |
| NS1-2/NS1-3 | 128–143/188–202 | YFV-NS1 | RKN GSF IID GKS RKE C/VNG KKS AHG SPT FWM-C | Anti-YFV-NS1-2/3 | Guinea pig |

into the extracellular space (Muller and Young, 2013). During natural YFV infection neutralizing antibodies are produced against the envelope protein (E) and the NS1 protein. However, knowledge about the specifics of antibody-mediated virus neutralization is limited, and investigation of these mechanisms would be of great value for development of epitope-based vaccines or antibody-based therapies (Dowd and Pierson, 2011; Pierson and Diamond, 2008).

The clinical presentation of fully developed YF is characterized by three stages (Monath, 2001). The first phase of infection, in which the patient is viremic, presents with unspecific symptoms. In the following remission phase the symptoms disappear and the patient may recover. Nevertheless, 15–25% of patients develop severe YF disease and enter the toxic phase which presents with typical symptoms such as hemorrhage and jaundice. Among patients reaching the toxic phase, 20–50% die from shock and multiple organ failure. In the later stages of infection the adaptive immune reaction clears the virus from the blood and YFV specific antibodies become detectable, probably conferring lifelong immunity (Monath, 2001).

Acute YF is confirmed by the direct detection of viral particles in the patient's blood during the viremic phase. The methods of choice are virus cultivation, plaque assay and the detection of the viral genome by PCR techniques such as qRT-PCR, isothermal or RPA assays (Weidmann et al., 2010; Bae et al., 2003; Drosten et al., 2002; Kwallah et al., 2013; Escadafal et al., 2014). However, the viremic phase might be restricted to a short period during the early stage of infection characterized by non-specific symptoms, making direct virus detection challenging if YF is not part of an initial differential diagnosis. As the disease progresses, indirect diagnosis is undertaken by detection of YFV specific antibodies using serological tests such as indirect immunofluorescence assays (IIFA), enzyme linked immune sorbent assays (ELISA) and plaque reduction neutralization assays (PRNT) (Niedrig et al., 2008; Kuno, 2003; Simoes et al., 2012). In serological assays, cross-reactivity to other flaviviruses and misdiagnosis should be considered (Mansfield et al., 2011; Houghton-Trivino et al., 2008). Another complication of early YF diagnosis is the potential diagnostic gap between the disappearance of viremia and the appearance of specific antibodies. In this regard, the detection of the NS1 protein has been shown to represent a useful alternative diagnostic target for other flaviviruses such as DENV, where the secreted form of the protein can be detected in the blood of patients between day 1 and 9 after the onset of symptoms (Alcon et al., 2002; Young et al., 2000). However, similar detection methods targeting the YFV-NS1 protein have not been developed until now and the lack of commercial antibodies targeting specific YFV proteins impedes the development of alternative diagnostic approaches. Commercial antibodies are available only for the detection of the YFV-E protein and full viral particles, whereas the specific detection of other viral proteins relies upon in-house antibodies and detection methods.

In order to extend the spectrum of specific tools for YFV research and diagnostics, this study produced and characterized new peptide antibodies for the detection of the YFV-NS1 and -C proteins.

## 2. Material and methods

### 2.1. Peptide selection

For the production of peptide antisera against YFV-NS1 and -C proteins, peptide sequences were selected using the Lasergene SeqMan Pro software (Version 8.1.5, DNASTAR, Madison, WI, USA). The protein sequences of 16 YFV strains were aligned in order to identify conserved regions (Accession numbers: U21055.1, U17066.1, DQ118157.1, NC_002031.1, DQ100292.1, X03700.1, X15062.1, AY640589.1, AY603338.1, AY572535.1, U21056.1, AF094612.1, U54798.1, DQ235229.1, AY968065.1 and AY968064.1). The generated consensus sequence was aligned with the protein sequences of other flaviviruses to select for non-conserved regions and to avoid possible cross-reactivity (Accession numbers: NC_001477 (DENV1), NC_001474 (DENV2), NC_001475 (DENV3), NC_001672 (TBEV), NC_007580 (St. Louis encephalitis virus), NC_001437 (JEV) and NC_001563 (WNV)). Resulting sequences were tested *in silico* for immunogenicity and surface exposure probability. Four peptides for immunization were synthesized (Eurogentec, Seraing, Belgium) (Table 1).

### 2.2. Immunization of animals and serum preparation

The production of polyclonal antisera against the synthetic peptides C and NS1-1 was performed by immunizing rabbits at the animal facility of the Robert Koch-Institute (Berlin, Germany). All animal experiments were approved by the responsible state ethics committee (LAGeSo Berlin, Germany). Pre-immune sera were collected before immunization and one rabbit was immunized by subcutaneous injection for each peptide. The initial immunization was performed with 140–220 μg peptide dissolved in complete Freund's Adjuvants (1:1). Subsequent immunizations were undertaken with 65–170 μg peptide dissolved in incomplete Freund's Adjuvants and saline in an overall volume of 1.5 ml every 2–6 weeks. The final bleed was conducted after the fifth booster immunization between days 160 and 166. Serum was collected by centrifugation of the blood for 12 min at 4 °C and 370 × *g* and stored at −20 °C.

Polyclonal antisera against the synthetic peptides NS1-2 and NS1-3 were commercially produced by immunizing guinea pigs, undertaken by Eurogentec (Seraing, Belgium). One animal was immunized with a combination of peptides NS1-2 and NS1-3, and two others were immunized with either peptide NS1-2 or NS1-3. All produced antisera are listed in Table 1.

### 2.3. SDS-PAGE, western blot (WB) and immunostaining of YFV proteins

Protein separation of culture supernatants or cellular extracts of YFV infected cells was performed under denaturing and half-native conditions by SDS-PAGE. For the denatured protein separation, samples were dissolved with 4× SDS-buffer containing β-mercaptoethanol (β-ME) (50 mM Tris, 40% glycerin, 8% β-ME, 4 g/l of bromphenol blue, 80 g/l of SDS; pH 6.8) and boiled for 5 min at 96 °C. For half-native conditions, samples were dissolved