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Expression and Purification of the SARS-CoV-2 Nucleocapside (Ncap) in *Escherichia coli*

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Abstract

Coronavirus-2 (SARS-CoV-2) causes severe acute respiratory síndrome and causes a global pandemic. Diagnosis of the disease is made through molecular and serological test, wich use SASR-CoV-2 antigens to detect the antibodies present in blood serum. The main antigens used are the spike protein (S) and the nucleocapsid (Ncap). Both proteins are obtanined in expression systems of eukaryotic and prokaryotic cells. In this work, the Ncap was expressed and produced in *Escherichia coli,* using a low-cost system and with possibilities of producing the antigen on a large scale. The nucleotide sequence was optimized to be expressed in *Escherichia coli*. After cell lysis, Ncap was located in the insoluble fraction of the cell free extract, forming inclusión bodies. Six consecutives histidines were fused at the C-terminus, wich gave rise the possibility of chemical interaction with divalent metals. The Ncap was therefore purified in immobilized metal affinity chromatography under denaturing conditions and exhibited stability for nine months. The results of the immunoblots with this viral antigen indicated its recognition by the será from patients previously diagnosed with SARS-CoV-2. The banding patterns of the inmunoblots obtained under hybrid conditions suggested proteolytic processing. The present work provides a strategy to produce the Ncap of SARS-CoV-2, with possibility of being used in the diagnosis of SARS-CoV-2.

Keywords: Nucleoprotein; Chromatography; Inmunoblots; SARS-CoV-2; Diagnosis

Introduction

According to the World Health Organization more than 772 million confirmed cases and 6 million deaths have been reported due to the severe acute respiratory syndrome type 2 coronavirus (SARS-CoV-2) until December 17, 2023 [1].

The SARS-CoV-2 is a single-stranded positive-sense RNA virus of 29.903 nucleotides and encodes 14 open reading phases, with the capacity for the synthesis of 27 proteins [2]. The main structural proteins of the virus are: the spike surface protein (S), the nucleocapsid (Ncap), the envelope protein (E) and the membrane protein (M). The S protein located on the surface of the virus during infection interacts with cellular angiotensin-converting enzyme (ACE-2) receptors [3,4].

The first SARS-CoV-2 genome sequence was reported in February 2020 [5], additionally circulating variants of interest have been identified as of

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28 June 2024: BA.2.86 and JN.1 and as of 19 July 2024 circulating variants under surveillance: JN.1.7, KP.2, KP.3, KP.3.1.1, JN.1.18 and LB.1 [6].

Ncap binds to viral RNA and forms a coiled-coil structure and is required for virus genome replication [7,8]. It is the structural protein synthesised in the highest proportion during infection [9-11]. It is highly immunogenic and antibodies against Ncap are detected in the early stages of infection [10,12-14]. Therefore, it is the antigen generally used for the diagnosis of SARS-CoV-2 infection [15].

The clinical detection of viral RNA is performed by reverse transcription-polymerase chain reaction (RT-PCR), however, molecular detection of RNA is expensive and requires strict biosafety measures, which is inconvenient in mass diagnostics. In addition, molecular techniques developed in conditions where patients suffer from heart disease, immunosuppression and diabetes give false negative results [16]. Notably, immunodiagnostics are less demanding, biologically safer and produce fewer false-negative results than molecular detection [17]. As an alternative, immunological methods have been used to complement molecular detection [18,19]. The immunological tests, which detect antibodies against SARS-CoV-2 in sera samples from infected patients, depend on selected antigens of the virus, such as spike protein (S) and nucleocapsid (Ncap) [20,21].

During infection, there is a time lag of approximately 5 days where molecular techniques, such as polymerase chain reaction (PCR), fail to detect the presence of SARS-CoV-2 and result in false negatives [22]. This contrasts with immunological detection, since antibodies; IgG and IgM, originating against Ncap are synthesised within 24 hours of infection and are maintained for 3-6 months after symptom onset [13,23].

Recombinant protein synthesis technology has made it possible to obtain and isolate Ncap and use it in immunoassays that confirm or rule out the existence of specific antibodies [4,24,25]. The immunological tests, as appropriate, contribute to the evaluation of the efficacy of the different drugs and vaccines available [26].

The Ncap is a 419 amino acid protein, which is divided into five regions, an intrinsically disordered N-terminal segment from amino acid 1 to 40 (N-arm), an N-terminal RNA-binding domain from amino acids 41 to 186 (NTD), a structurally disordered flexible central, connecting flexible region from amino acids 187 to 257 (LKR), with abundant serines and arginines. In this region, serines are post-translationally phosphorylated. A C-terminal dimerisation domain from amino acids 258 to 361 (CTD) and a structurally disordered segment from amino acids 362 to 461 (C-arm) [27-31]. The molecular weight of Ncap varies between 45 kDa and 60 kDa, depending on its post-translational modifications [32].

The Ncap is used in immunodetection techniques such as: chemiluminescent microparticle immunoassays [33], ELISA [34,35], Immunoblots [36] and lateral flow chromatography assays [37]. Several expression systems have been reported for the production of Ncap, including: production in mammalian embryonic cells (HEK-293), hamster ovary cells (CHO), insect cells such as *Spodoptera frugiperda* and in *Escherichia coli* [4,24,35,38-45].

In the present work, a strategy was implemented to express and purify the SARS-CoV-2 Ncap in *Escherichia coli*. Initially, a nucleotide sequence was deduced, which resulted in the optimisation of codon usage. The above procedure is a very convenient strategy to increase protein production levels in heterologous expression systems. The principle of codon optimisation is to avoid codons of low usage frequency, which tend to slow down the rate of gene translation [46,47]. Additionally, a low-complexity and low-cost gene induction system was designed. Ncap was purified to a high degree of homogeneity and stability.

Materials and Methods

Synthetis of pET20b-Ncap and confirmation of the construction

The nucleotide sequence of the SARS-CoV-2 was obtained from GenBank data base at acces code MN908947.3, which corresponded to the complete genome of the new coronavirus 2, from a strain designated as Wuhan-Hu-1 [48]. The Ncap coding sequence, comprising the region from nucleotide 28.274 to nucleotide 29.533, was then located in the virus genome. The sequence with 1.289 bp coded for a protein with a molecular weight of 45.6 kDa. To use the nucleotide sequence in an *Escherichia coli* expression system, the codon bias was optimised [49] using computer tools (http://genomes.urv.es/OPTIMIZER/) and was chemically synthesized (Genescript, USA). This modified sequence (GenBank ID: PQ009148.1) was cloned into a pET-20b (+) expression plasmid and the construct was designated pET20b- Ncap (Figure 1).

This vector fused the nucleotide sequence of Ncap to a sequence of six histidines at the C-terminal end that allowed purification by immobilised nickel affinity chromatography.

The *Thermus aquaticus* DNA polymerase was purchased from Promega (USA) and oligonucleotides with the designations T7 forward: AATACGACTCACTATAGG and T7 reverse: GCTAGTTATTATTGCTCAGCGG, were used in polymerase-mediated amplification (PCR) reactions to verify the correct construction of the vector.

Culture media, anti human IgG and resin for affinity chromatography

Peptone, casein, yeast extract and NaCl were purchased from Promega, (USA). Peroxidase-conjugated anti-

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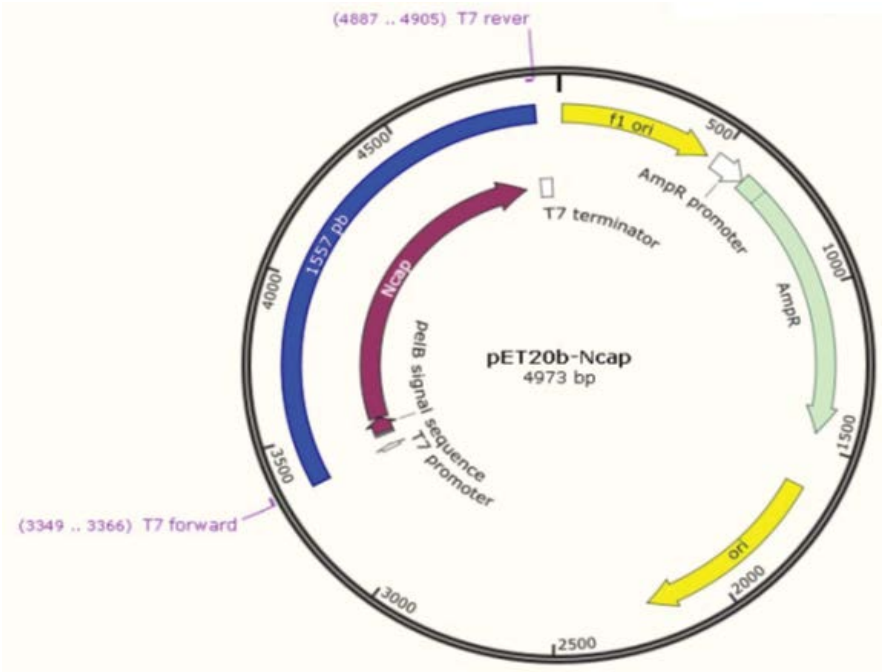

Figure 1: Map of the pET20b-Ncap vector: f1 ori: origin of replication of bacteriophage f1. AmpR: ampicillin resistance gene Ori: origin of bacterial replication. Pel B: signal sequence for export to the periplasm. RBS: ribosome-binding site. rop: plasmid copy control gene. Ncap: nucleoprotein gene. The 1.557 bp fragment resulting from amplification with the T7 forward and T7 reverse primers is schematised, the position of the primers is indicated in brackets.

human IgG was purchased from SIGMA (USA). Resin for immobilised metal affinity chromatography of nitrilotriacetic acid with nickel in an agarose matrix (Ni-NTA) was purchased from Invitrogen (USA).

Transformation and verification of the vector construction

The plasmid with the nucleocapsid gene insert designated pET20b-Ncap was resuspended in water to a final concentration of 0.5 ng/µl. 2.5 ng of pET20b-Ncap were used to transform to *E. coli* strain BL21 (DE3) purchased from New England Biolabs (USA). The transformation was performed according to the standardised methodology for this procedure [50]. Verification of transformation was done by seeding the transformation mixture in Petri dishes with lysogenyc broth (LB) containing 100 µg/ml of ampicillin and incubating at 37°C for 20 hr. A transformant colony was selected to verify the existence of the Ncap insert by a PCR reaction with T7 forward and T7 reverse oligonucleotides. The parameters used in the amplification reaction were: a 5 min cycle at 95°C; 32 denaturation cycles at 95°C for 1 min, a hybridisation cycle at 45°C for 3 min, an extension cycle at 72°C for 1 min, and a final extension at 72°C for 5 min. The fragment was visualised and identified by electrophoresis on agarose gels.

Expression of Ncap in *E. coli.* BL21 (DE3)

A transformant colony of *E. coli.* BL21 (DE3), was inoculated into 3 ml of LB supplemented with ampicillin (100 ug/ml) and grown at 37°C with shaking at 150 r.p.m. for 16 hr. It was then diluted 1:100 in 50 ml of LB with ampicilin and 1 % of lactose and grown at 37°C with shaking at 150 r.p.m. for 16 hrs. The culture was centrifuged at 12.000 g for 15 min at 4°C, then the cell pellet was stored at -20°C.

Purification of Ncap

Hybrid and denaturing conditions

Cells were thawed and resuspended in 10 ml of 100 mM phosphate buffer, pH 8, lysozyme (1 mg/ml) and phenylmethylsulphonylfluoride (1mM). Incubated at room temperature for 60 minutes and frozen for 16 hours, thawed, centrifuged and the viscous sediment separated from the supernatant. In the hybrid purification condition, urea was added to the above sediment until a final concentration of 6M was reached. It was shaken for 16 hours and centrifuged. NaCl and imidazole were added to the supernatant to final concentrations of 300 mM and 10 mM respectively. The supernatant was mixed with 200 µl of Ni-NTA resin, equilibrated with 100 mM phosphate buffer, pH 8, 300 mM NaCl, 10 mM imidazole and 6 M urea. The mixture of the supernatant with the resin was shaken for 1 hr at room temperature. The resin was packed into a column and 5 volumes of 100 mM phosphate buffer, pH 8, 300 mM NaCl, 10 mM imidazole and 6M urea were added. Subsequently, 5 volumes of buffer without urea were passed through the resin.

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The Ncap was then eluted with an increasing imidazole gradient of 5 column volumes for each fraction of; 250 mM, 500 mM and 1000 mM in 100 mM phosphate buffer, pH 8 and 300 mM NaCl. In the denaturing condition, 5 volumes of 100 mM phosphate buffer, pH 8, 300 mM NaCl and 6 M urea were applied. The fixed Ncap was eluted with a gradient of pHs in the same buffer of 7; 6; 5.3; 4 and 2 respectively.

Polyacrylamide gels and immunoblots

The Ncap was visualised using 15% S.D.S. polyacrylamide gels which were stained with Coomasie blue according to the methodology reported for this procedure [51]. For immunoblots, after completion of electrophoresis on polyacrylamide gels, Ncap was transferred to polyvinylidene fluoride (PVDF) membranes purchased from Milipore (USA), in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at a constant current of 2 mA/cm^2 for 30 min, using a semi dry transfer kit, purchased from Bio-Rad (USA) for 45 min. At the end of the transfer, the membrane was immersed in 20 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 0.05 % Tween 20 with 5% non-fat milk, for 1 hr at 37°C, with gentle agitation.

The sera to be tested was then added at a final dilution of 1:50. Sera from patients diagnosed with SARS-CoV-2 using the COVID-19 kit purchased from Abbott (USA) and sera from healthy volunteers were used. The mixture was incubated at 37ºC with gentle agitation for 30 min. The membrane was then washed 3 times with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl with 0.05% Tween 20 and incubated with a peroxidase-conjugated anti-human IgG antibody purchased from Sigma (USA) at a dilution of 1:1000 for 1 hr at 37 °C with gentle agitation. The membrane incubated with the anti-human IgG was washed 3 times with the same buffer, and finally the membrane was washed in the same solution, without Tween 20. The reaction band, originating from the recognition of the anti-IgG, was detected by immersing the membrane in 5 mM diaminobenzidine and 0.2 % H_2O_2.

The sera from patients diagnosed with SARS-CoV-2 (N=11) and negative sera (N=7) were collected in a clinical laboratory, and volunteers provided written consent prior to sample request for their participation in the study. The study and use of samples was done following the guidelines established in the Helsinki declaration for human research outlined in the FONACIT bioethics and biosafety code [53].

Results

Verification of the synthetic construction of the Ncap gene

Figure 2 shows the results obtained in the PCR, performed to verify the correct construction of the vector. A band of 1.557 bp was obtained, corresponding to the length of the fragment, which included part of the vector and the Ncap sequence (Figure 2).

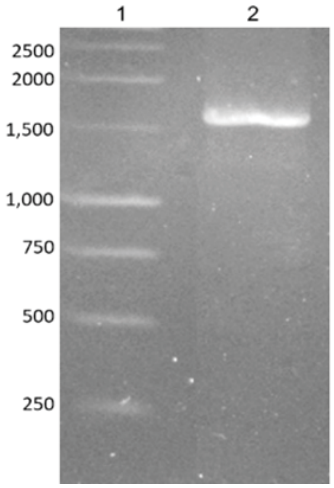

Figure 2: Agarose gel (2%) analysis of the product of PCR reaction with the specific oligonucleotides for the amplification of the segment of the pET20b-Ncap vector that included the Ncap gene. Lane 1: Molecular weight marker (Invitrogen, USA). Lane 2: Product of the amplification reaction.

Expression and solubility of Ncap in *E. coli*. BL21 (DE3)

In Figure 3 a polyacrylamide gel with the fractions obtained from a culture of BL21 (DE3) with the plasmid pET20b-Ncap, with 1 % lactose, is shown. A band with a molecular weight of approximately 50 kDa, corresponding to the Ncap in the cell extract, was obtained and located in the insoluble fraction, after cell disruption, forming inclusion bodies (IB).

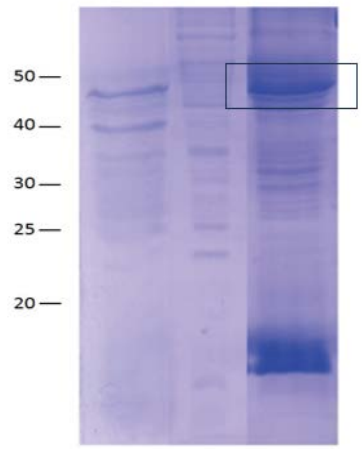

Figure 3: Electrophorogram analysis of the SARS-CoV-2 Ncap protein. SDS-PAGE (15%) of the expressed Ncap. Lane 1: cell extract. Lane 2: supernatant of cell disruption. Lane 3: insoluble fraction of cell disruption. 50 µg of protein per lane were used. Ncap is included in the inset.

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Purification of Ncap and its use in immunoblots

The location of Ncap in IB precluded its isolation under native conditions. Therefore, purification was performed using hybrid and denaturing purification methodologies.

Figure 4 shows the results obtained on a polyacrylamide gel with the fractions from the hybrid purification and the immunoblots made with the purified Ncap fraction. Most of the Ncap was homogeneously eluted with 500 mM imidazole. Immunoblots made with the 500 mM eluted fraction indicate reaction with SARS-CoV-2 positive serum (Figure 4B: lane 1), however the banding pattern suggests the appearance of additional reaction bands. It should be noted that the Ncap isolated under these conditions degraded and the protein band disappeared after 72 hr, despite storage at 4°C, making it impossible to use it in further experiments.

Due to the instability of the isolated Ncap under hybrid conditions, it was purified under denaturing conditions. In this procedure, 6 M urea was incorporated, both in the solution used to dissolve the IB, as well as in the solutions of the immobilised metal affinity chromatography. The results are shown in Figure 5. Ncap eluted with a high degree of homogeneity at pH 5.3. Furthermore, immunoblots made with this fraction exhibited mainly one reaction band with very little evidence of additional bands. Notably, Ncap isolated under denaturing conditions was stable over time, and it was possible to use samples stored at 4°C as antigen after 9 months in immunodetection assays.

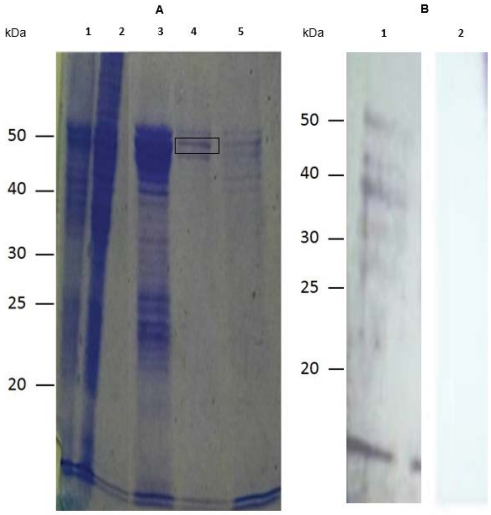

Figure 4: Analysis of Ncap obtained under hybridised conditions. A: SDS-PAGE (15%) of the purified Ncap fractions. Lane 1: extract of cells cultured with 1 % lactose. Lane 2: solubilised IB. Lanes 3, 4 and 5: fractions eluted with 250 mM, 500 mM and 1000 mM imidazole respectively. 50 µg of protein per lane were used Molecular weight markers in kDa are indicated on the left. Ncap is included in the inset. B: Immunoblot of the 500 mM imidazole eluted fraction with diagnosed sera positive and negative for SARS-CoV-2. Lane 1: serum diagnosed with SARS-CoV-2. Lane 2: Control serum. Molecular weight markers in kDa are indicated on the left.

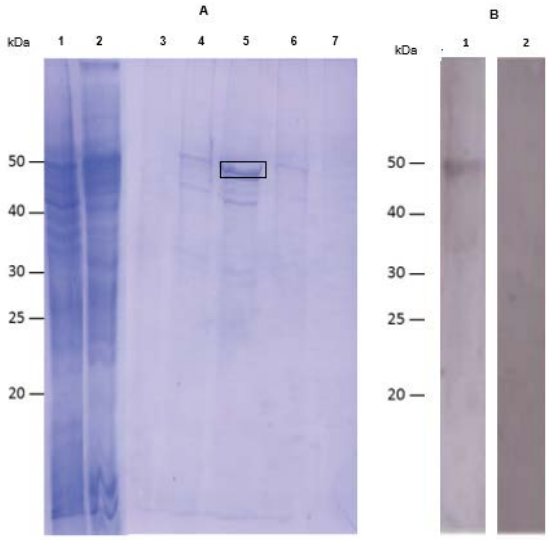

Figure 5: Analysis of Ncap obtained under denaturing conditions. A: SDS-PAGE (15%) of the purified Ncap fractions. Lane 1: extract of cells cultured with 1 % lactose. Lane 2: solubilised IB. Lanes 3, 4, 5 and 6: elutions at pHs of 7; 6; 5,3; 4 and 2, respectively of the resin bound material. Ncap is included in the inset. 50 µg of protein per lane were used. On the left are the molecular weight markers in kDa. B: Immunoblot of the eluted fraction at pH 5.3 with positively and negatively diagnosed sera. Lane 1: serum diagnosed with SARS-CoV-2. Lane 2: control serum. On the left are the molecular weight markers in kDa.

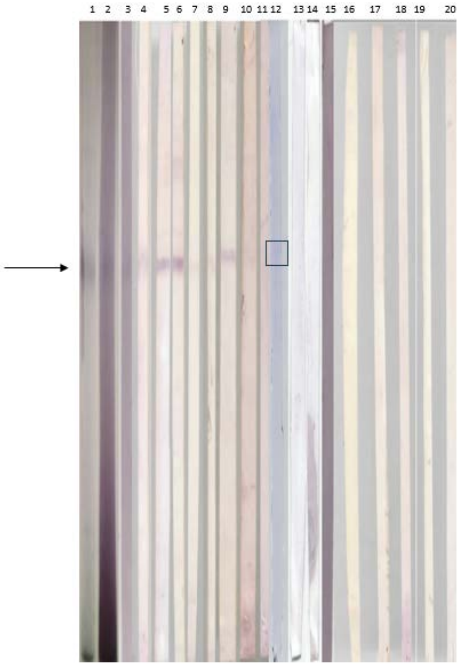

Figure 6: Immunoblots of Ncap as antigen and SARS-CoV-2 positive and control sera samples. Lanes 1 to 11 show immunoblots obtained with sera from patients diagnosed with SARS-CoV-2, lane 12 shows a polyacrylamide gel with the Ncap band, framed in a box. Lanes 13 to 20 are immunoblots with sera diagnosed negative for SARS-CoV-2. The arrow indicates the reaction band.

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Immunoblots with sera from COVID-19 positive and negative patients

The fraction eluted under denaturing conditions at pH 5.3 was used as antigen in immunodetection assays. Immunoblots with sera from patients diagnosed with SARS-CoV-2 and sera from healthy volunteers are shown in Figure 6. Reaction was detected with sera from patients diagnosed positive for SARS-CoV-2. The difference in intensity of the reaction bands suggests differences in antibody titres between SARS-CoV-2 positive patients. Reactions with SARS-CoV-2 positive sera, shown in lanes 4 through 11, were made with Ncap samples with 9 months storage at 4°C.

Discussion

By changing the codon bias, adjusting them to that used by *Escherichia coli*, the nucleotide sequence of the Ncap of SARS-CoV-2 was synthesized. The strategy was effective and the production of Ncap in *Escherichia coli* could be verified.

The genetic induction of Ncap is generally mediated by the inclusion of IPTG in the cell culture [24,25,39,53]. IPTG is expensive and as an alternative, Ncap gene expression has been carried out in autoinduction medium [39,40,43,54]. Similarly, autoinduction medium is expensive given the variety of its components [55], so induction was replaced by IPTG by culturing the cells in LB with 1% lactose, which effectively induced Ncap production. Lactose is less expensive and toxic, it is also used as a source of carbon and energy and simultaneously acts as an inducer of genetic expression [55, 57].

The Ncap accumulated in IB, which prevented its isolation under native conditions, so we proceeded to isolate the Ncap under denaturing conditions. In this procedure, a fraction of the Ncap was obtained with an estimated of; 90 % of homogeneity that exhibited stability for at least 9 months. The estimated yield taking as reference the intensity of the Ncap band in polyacrylamide gels with S.D.S. of the cell lysate, was 2,5 mg/lt of culture.

On the other hand it was also proceeded to purify the Ncap under hybrid conditions. Under these conditions the purification was initiated by solubilization of the IB and fixation to the metal affinity resin in the presence of 6 M urea. Homogeneous form elution was achieved with 500 mM imidazole in the absence of 6 M urea. Immunoblots performed with this fraction presented a heterogeneous reaction pattern. Also under hybrid conditions it was possible to purify to homogeneity the Ncap, but it turned out to be unstable, upon degradation after 72 hrs of its storage at 4°C. The above observation agrees with repeated reports of instability of the SARS-CoV nucleocapsid, given its susceptibility to proteolysis [35,58], indeed the pattern of heterogeneous bands in the immunoblot must have been produced by Ncap proteolysis. The above results contrast with those obtained using the Ncap coming from denaturing conditions. Thus the immunoblots obtained with the fraction of denaturing conditions, originate predominantly one reaction band, with slight indications of additional bands, suggesting decreased proteolytic degradation activity.

It is noteworthy that there are reports of the autoproteolytic processing of SARS-CoV-2 nucleoprotein [59,60]. Specifically, self-proteolytic processing has been demonstrated *in vitro*, by incubating the isolated nucleoprotein in the presence of protein inhibitors and, *in vivo*, in nucleoprotein transfectants obtained with HEK293T cells. In both systems the same proteolytic processing pattern appears. The authors define self-proteolytic processing as spontaneous proteolysis, which depends on the conformation of the polypeptide, and the production of 5 different proteoforms has been reported [60]. In this report, the term proteoforms is adopted, to designate to the different molecular forms of peptides derived from the translation of a gene, which result as a consequence of post-translational modifications or proteolytic processes [61].

Additionally it has been shown that the highest immunogenicity resides in the terminal C domain and a proteoform with the terminal C domain, constitutes one of the products of self-proteolysis. Further, it has been proposed that the proteoform with the terminal C-domain, would serve as a decoy to attract antibodies and thus evade their action, during the virus lytic cycle [60].

The procedure of isolation of the Ncap under denaturing conditions with 6M urea and its storage under the same conditions, must have eliminated the possibility of autoproteolysis, whose mechanism additionally is unknown [60].

Despite the fact that Ncap was isolated under denaturing conditions, its renaturation was not required in the detection assays via immunoblots. Renaturalization followed by isolation under denaturing conditions has been an experimental scheme that has generally been followed for the utilization of Ncap in immunodetection experiments [58,62]. Overall, recombinant Ncap has been found to be associated with RNA, which is consistent with its role in virus assembly, however such association prevents its recognition by antibodies present in sera from positive SARS-CoV-2 patients [62,63]. It has been proposed that the association of nucleic acid with Ncap blocks the recognition of epitopes by antibodies. One of the strategies followed to remove Ncap-associated nucleic acid has consisted of its isolation under denaturing conditions in the presence of 6 M urea and its subsequent renaturation for immunodetection assays [41,58,62].

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Therefore, the procedure followed for obtaining the Ncap in the present work, under denaturing conditions not only enabled the removal of the nucleic acid, and the increase of its stability, additionally also favored its immune recognition, as evidenced in the immunoblots (Figure 6). The lack of requirement for its renaturation may have been a consequence of the recognition of linear epitopes rather than conformational epitopes, additionally it is possible that given the flexibility and lack of ordered structures in most of the Ncap molecule [24,60] during the experimental procedure, the molecule has adopted a conformation that allowed it to be recognized by antibodies. The above hypotheses would of course require additional experiments for checking.

In all reports using the solubilized fraction of the IB, and an immobilized Ni-NTA resin chromatography, additional steps are included to obtain a homogeneous fraction [41,58,62,63], the methodology followed in this work, did not require additional steps and in a filtering step in immobilized nickel resin a fraction with a 90 % homogeneity was obtained. The high efficiency in the isolation of Ncap, must have been consequence of its accumulation in IB and the use of a reduced amount (200 µl) of resin with immobilized nickel. The Ncap during chromatography under denaturing conditions should have saturated most of the available sites present in the resin of ANT-Ni, allowing the binding of few contaminating proteins.

The formation of IB is frequently achieved in the production of recombinant proteins, with high rate of translation. They are mainly constituted by the overproduced protein [35,64]. In addition proteins accumulated in IB are stabilized and resistant to proteolytic attack [65,66]. The above features therefore, must have contributed to the efficient isolation of the Ncap.

In conclusión, the present work demonstrated; the production of Ncap in *E. coli* and its subsequent purification under denaturing conditions, which resulted in an increase in its stability and enabled its use in immunodiagnostic assays. The procedure is relatively simple and low-cost and could be carried out on a large scale to obtain the antigen that could be used in the development of serological diagnostic methods for SARS-CoV-2.

Conflict of Interest

The authors declare they have no conflicto of interest.

Aknowledgements

Project 2007001425 FONACIT “Development and/or Suitability of Inputs to be Applied to Cases for Serological Diagnosis for Infectious Infectious Diseases”.

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