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Original Article

Evaluation of an *E. coli*-expressed spike protein-based in-house ELISA system for assessment of antibody responses after COVID-19 infection and vaccination

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Abstract

Evaluating long-term immunity after COVID-19 infection and vaccination is critical for managing potential outbreaks. The aim of this study was to develop a cost-effective in-house enzyme-linked immunosorbent assay (ELISA) based on *Escherichia coli*-expressed SARS-CoV-2 spike protein (E-S1) for antibody detection and to evaluate its performance. The system was validated by comparing the in-house ELISA results with those obtained using a commercial ELISA with HEK293-expressed spike protein (H-S1). Recombinant SARS-CoV-2 spike protein was produced in *E. coli*, purified, and validated for antigenicity via ELISA. Indirect ELISAs with both E-S1 and H-S1 antigens were performed on 386 serum samples from COVID-19 survivors, vaccinated individuals, and pre-pandemic controls collected at different time points. The E-S1 ELISA showed a statistically significant but weak correlation with H-S1 ELISA across all samples (r=0.205; p=0.0001). Stronger correlations were observed among vaccinated individuals with prior infection on day 90 (r=0.6017; p<0.001) and in naïve vaccine recipients on day 30 (r=0.5361; p=0.0003). Pre-pandemic sera from a rural population in Sumba Island exhibited high background reactivity in E-S1 ELISA, likely due to anti-*E. coli* antibodies, while urban pre-pandemic sera from Jakarta showed a stronger correlation with H-S1 ELISA. This suggests potential regional or immune background differences influencing assay performance. Although E-S1 retained antigenic properties, its diagnostic utility is limited by non-specific reactivity and reduced sensitivity compared to H-S1. In conclusion, *E. coli* expression systems may not be ideal for producing spike protein-based ELISA antigens specific to SARS-CoV-2. Alternative expression systems, such as human or baculovirus, could enhance diagnostic accuracy and specificity for COVID-19 antibody detection.

Keywords: Capture antigen, COVID-19, ELISA, *E. coli* expression system, antibody detection

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Introduction

The COVID-19 pandemic, caused by the novel coronavirus SARS-CoV-2, has significantly impacted global health, resulting in over 767 million confirmed cases and nearly 7 million deaths worldwide as of June 2023 [1-4]. SARS-CoV-2 infection can result in severe respiratory complications, including pneumonia and respiratory failure, and has been associated with a mortality rate of approximately 1% among symptomatic cases [5-7]. While real-time polymerase chain reaction (RT-PCR) remains the gold standard for diagnosing acute SARS-CoV-2 infection by detecting viral genomic material in respiratory specimens, it does not provide insight into long-term immunity [3,8]. Assessing antibody responses following infection or vaccination is crucial for understanding levels of population immunity and informing strategies to mitigate future outbreaks [5].

Vaccination campaigns have been widely implemented. However, serological testing remains crucial for evaluating immunity levels and supporting public health surveillance [9]. Enzyme-linked immunosorbent assay (ELISA)-based serological assays, which detect antibody-targeting SARS-CoV-2-specific proteins such as the spike (S) protein, receptor-binding domain (RBD), and nucleocapsid protein (NP), have played a critical role in assessing immune responses following infection and vaccination. Among these targets, the S protein, specifically S1 subunit, exhibits specificity to SARS-CoV-2 and serves as a primary antigen for neutralizing antibodies, making it an ideal candidate for seroepidemiological studies [3,8,10]. Developing accurate and cost-effective serological assays is important, especially in resource-limited settings, where commercial ELISA kits may be prohibitively expensive [11].

Most commercial ELISA kits utilize spike proteins produced in mammalian systems, such as HEK293 cells, which provide proper folding and glycosylation, resulting in high sensitivity and specificity [12]. However, producing these mammalian-derived proteins is expensive, presenting significant challenges for widespread implementation, particularly in resource-limited settings [13]. Developing an in-house ELISA system using microbial expression systems, such as *Escherichia coli*, could significantly reduce costs, making it accessible for broader use in developed and developing regions [14].

E. coli is one of the most widely used bacterial systems for recombinant protein production due to its rapid growth rate, ability to achieve high-density culture, and affordability [15]. Additionally, *E. coli* allows for straightforward genetic manipulation, rapid transformation, and high protein yield, making it an attractive alternative to mammalian expression systems [16]. However, there are potential limitations to using *E. coli*-expressed proteins in immunoassays. Unlike mammalian systems, *E. coli* lacks the ability to perform post-translational modifications, such as glycosylation, which can affect protein folding and epitope presentation [17]. Consequently, there is a risk that *E. coli*-expressed spike proteins may not fully replicate the antigenic structure required for accurate antibody detection, potentially compromising assay performance [18].

Several studies have investigated the use of *E. coli* for producing SARS-CoV-2 proteins, yielding mixed results [19-21]. While some studies have shown that *E. coli*-expressed spike or NPs can elicit antibody responses in ELISAs, the sensitivity and specificity of these assays are often diminished due to differences in protein structure [22]. Nevertheless, with the significant cost reduction offered by *E. coli* expression systems, there is an urgent need for further study on their feasibility for producing SARS-CoV-2 antigens for serological assays, particularly in low-income regions where access to diagnostic testing remains a challenge [10,23,24].

Serological testing plays a crucial role in understanding and managing the COVID-19 pandemic. However, current commercial assays can be expensive and difficult to access, especially in resource-limited settings. This study evaluates the performance of a recombinant SARS-CoV-2 S1 spike protein, expressed using a cost-effective *E. coli* expression system (E-S1), as the capture antigen in an in-house ELISA for detecting SARS-CoV-2 antibodies. The performance of the E-S1 ELISA was compared to that of a commercial ELISA utilizing a HEK293-expressed S1 protein (H-S1) across a diverse cohort of serum samples from COVID-19 survivors, vaccinated individuals, and pre-pandemic controls. The correlation between the E-S1 and H-S1 assays was also assessed. Therefore, the aim of this study was to develop a

recombinant SARS-CoV-2 S1 spike protein using an E-S1 system and evaluate its performance as a capture antigen in an in-house ELISA.

Methods

Study design

Serum samples were collected as part of a cohort study conducted at Dr. Tadjuddin Chalid Hospital, Makassar, Indonesia, from April to December 2021. This study involved COVID-19 recovery patients and vaccinated individuals participating in a vaccine development study, all provided signed informed consent [25].

The inclusion criteria required participants to have recovered from COVID-19, confirmed by a negative PCR test after infection. Blood samples were collected serially from these individuals up until day 90. In addition, vaccine recipients who had received two doses of whole-inactivated vaccine were included in this study. This group comprised individuals without a history of COVID-19 (naïve vaccine) and those who experienced a breakthrough infection. Blood samples were collected at days 0, 30, and 90 after the second vaccine dose to capture distinct stages of antibody response following viral exposures or vaccination. Blood samples were extracted only on day 0 for the breakthrough infection group. Day 0 represented the immediate post-vaccination phase, providing a baseline measurement of the antibody response shortly after recovery or vaccination completion. Day 30 corresponded to the peak antibody response, typically when immune responses are expected to reach their highest levels following the second dose. By day 90, antibody levels commonly start to decline, allowing for the assessment of the immune response over an extended period.

In this study, a total of 386 samples were analyzed, consisting of 156 samples from day 0 (35 samples from COVID-19 survivors, 52 samples from pre-infected vaccine recipients, 41 samples from naïve vaccine recipients, and 28 samples from COVID-19 breakthrough infections), 110 samples from day 30 (19 samples from covid-19 survivors, 50 samples from pre-infected vaccine recipients, and 41 samples from naïve vaccine recipients), 90 samples from day 90 (14 samples from covid-19 survivors, 41 samples from pre-infected vaccine recipients, and 35 samples from naïve vaccine recipients); and 30 samples from the pre-pandemic era as presented in **Table 1**. All samples were subjected to ELISA using E-S1 and H-S1.

Human serum collection

Blood samples were collected from each participant, and sera were separated using a serum separator tube (BD Vacutainer™ Venous Blood Collection Tube: SST™ serum separation tube hemogard) followed by 10 min centrifugation at 3,500 rpm (Centrifuge 5702 R, Eppendorf, Hamburg, Germany) at the Hasanuddin University Medical Research Centre (HUMRC) of Universitas Hasanuddin Hospital, Makassar, Indonesia. All serum samples were stored at -80°C following centrifugation until further analysis for antibody detection using ELISA. In addition, pre-pandemic serum samples collected in 2017 from Sumba Island, East Nusa Tenggara Province, Indonesia, were included as the negative control group (**Table 1**). They represented individuals with no known COVID-19 exposure, establishing a baseline for antibody levels in an unexposed population. These samples are crucial for validating the ELISA assay's specificity, ensuring reliable differentiation between valid positive results in the study groups and any background reactivity.

Table 1. Number of samples used for enzyme-linked immunosorbent assay (ELISA)

Group	Day 0 (n=186)	Day 30 (n=110)	Day 90 (n=90)
COVID-19 survivors (group a)	35	19	14
Pre-infected vaccine recipients (group b)	52	50	41
Naïve vaccine recipients (group c)	41	41	35
Breakthrough infection (group d)	28		
Pre-pandemic (group e)	30		

Development of recombinant protein spike 1

The E-S1 was developed at the Laboratory of Vaccinology and Applied Immunology, Kanazawa University, Ishikawa, Japan. The pcDNA3.1 plasmid encoding the Wuhan-Hu-1 SARS-CoV-2 spike protein (S) was obtained from Addgene (#145032) [26,27]. To amplify the target S1 sequence (residues 14-685), a specific primer pair, p2020C-S1-F1 (#986; CACCGAATTCCAGTGCGTGAACCTGCC) and p2020C-S1-R3 (#992; TGGGC TCGAGCCTGGCTCTCCTTGGGGAGTTTGT) was used for the PCR amplification of SARS-CoV-2 S14-685 (Spike 1). The PCR product was initially cloned into the pENTR-TOPO vector (Invitrogen, San Diego, USA) and subsequently digested with EcoRI/XhoI restriction enzymes for subcloning into the pET32b-sPfCSP vector [28]. The resulting expression plasmid was transformed into *E. coli* BL21 (DE3) cells for protein production.

Induction tests were performed on multiple transformed colonies, and colony 1 was selected for large-scale protein production due to its high expression levels and solubility profile. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that most of the recombinant S1 protein was found in the insoluble fraction, requiring solubilization for further purification.

Protein purification was conducted following established protocols [29]. Briefly, cell cultures were grown for three hours at 37°C, followed by induction with 1 mM isopropyl 1-thio-β-d-galactopyranoside for another three hours. After harvesting the cells, the E-S1 protein was solubilized in guanidine hydrochloride (Gu-HCl) to recover the protein from inclusion bodies. The protein was then purified by chromatography using nickel-nitrilotriacetic acid (Ni-NTA) agarose chromatography (Qiagen) with elution achieved by applying an imidazole gradient (from 10 mM to 1 M) to remove non-target proteins and ultimately elute the target protein sequentially. The final elution was performed using 1 M imidazole in a tris-buffered saline buffer to achieve maximum purity.

To confirm the antigenicity of the purified E-S1 protein, an ELISA was conducted using an anti-S1 monoclonal antibody (mAb). The purified E-S1 protein was coated onto ELISA plates (Costar EIA/RIA polystyrene plates, Corning Inc., New York, USA) with a serial dilution starting from 1:1000, at concentrations of 4 µg/mL and 40 µg/mL. Plates were then incubated with the commercial anti-Spike mAb (Sino Biological, Beijing, China) or anti-Spike S1 mAb (Sino Biological, Beijing, China) to validate the antigenic properties of the recombinant S1 protein.

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISAs were carried out utilizing the E-S1 and the commercial H-S1 protein produced in the human embryonic kidney (HEK293) (Sino Biological, Beijing, China) as the antigens. All samples were subjected to ELISAs with both antigens. The 96-well microplate (Costar EIA/RIA polystyrene plates, Corning Inc., New York, USA) was coated with 0.2µg/mL of recombinant protein E-S1 or H-S1 per well and incubated overnight at 4°C. The next day, serum samples (diluted 1:100 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA)) were incubated on the plates for one hour after previously blocked with 1% BSA in PBS solution. After incubation, the plates were washed, and samples were incubated with monoclonal antibodies that recognized the Fc domain of human IgG conjugated with horseradish peroxidase (HRP) (Bio-Rad Lab Inc., Tokyo, Japan). After washing, 100 µL per well of the substrate solution, composed of H_2O_2 and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate), was added to each well and incubated for 30 min at room temperature. The absorbance of the developed color was measured at 414 nm using a microplate reader (Multiskan FC, Thermo Scientific, Shanghai, China).

Statistical analysis

GraphPad Prism version 9.0 (GraphPad Software Inc., San Diego, USA) for Mac OS was used to conduct statistical analysis. Correlations between optical densities (ODs) of E-S1 ELISA and H-S1 ELISA were evaluated using Spearman's rank correlation test. In addition, the Mann-Whitney U test was used to analyze the significance of the difference in ODs between the two groups. A p-value of <0.05 was considered statistically significant.

Results

Design, expression, and evaluation of E-S1 antigen

The E-S1 antigen was designed to include the S1 subunit (residues 13–525), covering the RBD, which is critical for immune recognition (**Figure 1A**). This design was chosen to capture the key antigenic determinants relevant to SARS-CoV-2 immunity.

The S1 gene was cloned into an expression vector and transformed into *E. coli* BL21 (DE3) cells, with protein expression induced using IPTG (**Figure 1B**). Following expression, the protein was primarily found in the insoluble fraction, necessitating purification under denaturing conditions. The purification process utilized imidazole elution steps with increasing concentrations from 10 mM to 1 M (lane 4–14) to isolate the target protein from contaminants. SDS-PAGE analysis revealed a prominent band at approximately 100 kDa in the higher elution fractions (100 mM and 1 M), which aligns with the expected molecular weight of the S1 protein, confirming successful isolation.

To evaluate the antigenicity of the E-S1, indirect ELISAs were performed using two different SARS-CoV-2-specific antibodies: the SARS-CoV-2 Spike mAb and the SARS-CoV-2 S1 mAb. The E-S1 protein was coated on ELISA plates, and serial dilutions of the antibodies were applied to assess binding efficiency. The result illustrates the serial dilution setup, with color intensity reflecting binding strength at various dilution levels (**Figure 1C**).

The SARS-CoV-2 Spike mAb showed a strong dose-dependent binding response to the E-S1 protein (**Figure 1D**). This antibody exhibited detectable binding up to a dilution of approximately 1:38,462 at a coating concentration of 40 µg/mL, with absorbance values decreasing as the dilution increased. At a lower concentration of 4 µg/mL, the detection limit was around 1:1,538, indicating reduced sensitivity. The absorbance threshold was set at 0.15 to establish a baseline for detection across samples.

In contrast, the SARS-CoV-2 S1 mAb showed significantly higher sensitivity and broader reactivity with the E-S1 protein (**Figure 1E**). At a 4 µg/mL concentration, detectable binding was sustained up to a dilution ratio exceeding 1:2,048,000, indicating a strong affinity for the E-S1 antigen. Even at a higher concentration of 40 µg/mL, this antibody maintained robust binding with detection up to a dilution ratio of 1:2,000,000, highlighting its superior sensitivity.

Evaluation of in-house ELISA with *E. coli*-expressed S1 antigen (E-S1)

The in-house ELISA system using the E-S1 antigen was compared with a commercial H-S1 antigen to assess their ability to detect antibody responses across COVID-19-infected individuals, vaccinated individuals, and pre-pandemic controls. The H-S1 antigen yielded significantly higher OD values for all samples than the E-S1 antigen. The median OD for H-S1 was 1.741 (Interquartile range (IQR): 1.094–2.090), compared to 0.783 (IQR: 0.567–1.069) for E-S1, showing a highly significant difference (p<0.0001) (**Figure 2A**). This indicates a stronger antibody response detected by the human cell-derived H-S1 antigen across the sample set.

At day 0, H-S1 continued to show a markedly higher median OD of 1.774 (IQR: 1.091–2.028) versus 0.6935 (IQR: 0.512–0.959) for E-S1, with a significant difference (p<0.0001) (**Figure 2B**). This demonstrates the immediate superior sensitivity of the H-S1 antigen in antibody detection post-infection or vaccination. Moreover, by Day 30, the H-S1 antigen maintained its superiority in antibody response detection, with a median OD of 1.759 (IQR: 0.742–2.105) compared to 0.721 (IQR: 0.539–0.999) for E-S1. The difference was statistically significant (p<0.0001) (**Figure 2C**), indicating a persistent lead of the H-S1 antigen. Finally, on Day 90, the H-S1 antigen's median OD was 1.725 (IQR: 1.408–2.309), which remained significantly higher than the E-S1 antigen's median OD of 1.04 (IQR: 0.816–1.326) (p<0.0001) (**Figure 2D**). This suggests that H-S1 continues to be more effective in detecting long-term antibody responses.

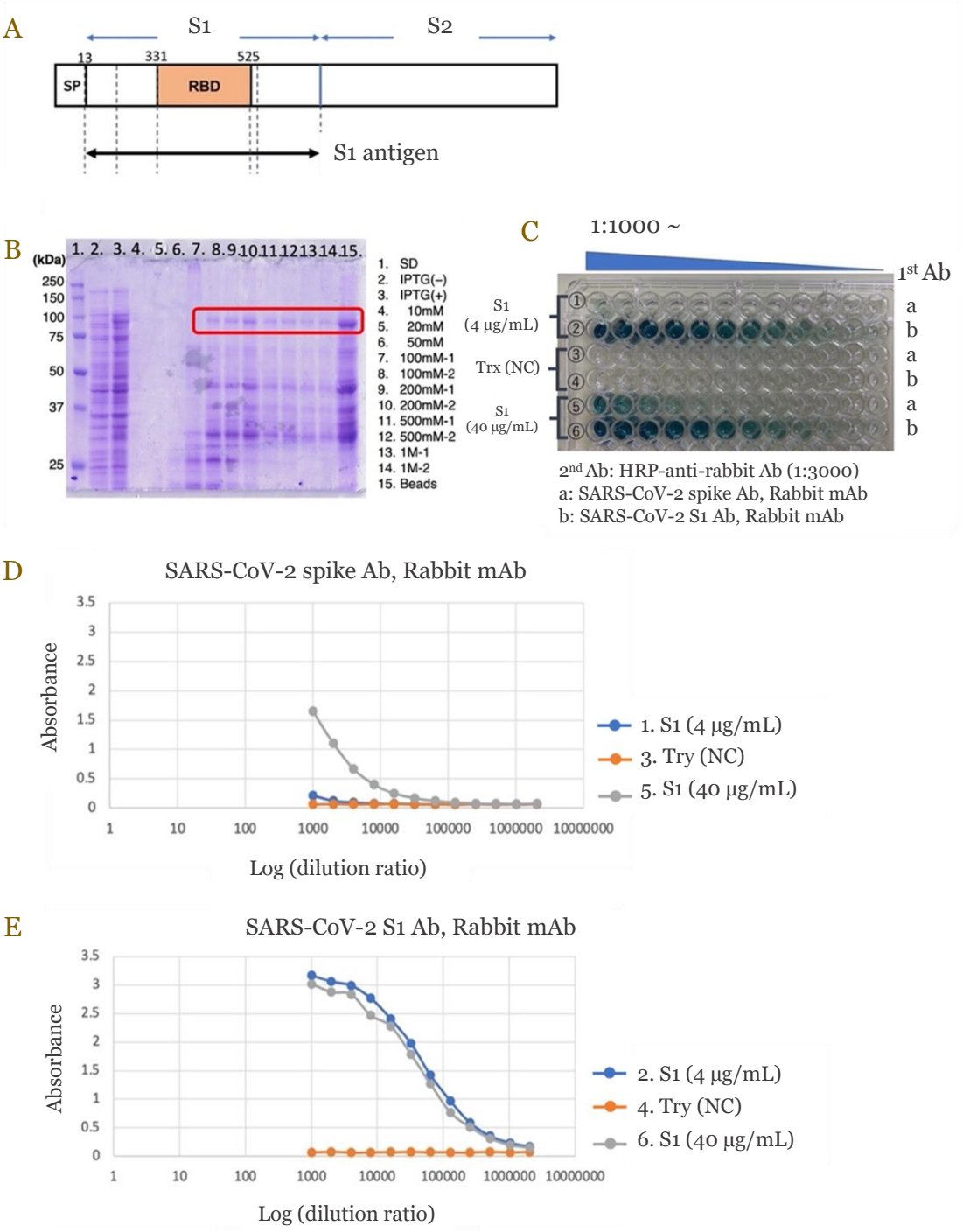

Figure 1. Design of SARS-CoV-2 antigen for production using *Escherichia coli* expression system (E-S1). (A) The domain of the spike protein-encoding gene selected for antigen production; (B) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the resulting protein from each step in the purification process. The expected protein is highlighted in the red square. Lane 1 represents the molecular weight marker; (C) antigen optimization using anti-SARS-CoV-2 spike antibody and spike S1 antibody; (D) enzyme-linked immunosorbent assay (ELISA) results for the *E. coli*-expressed S1 antigen using the SARS-CoV-2 spike monoclonal antibody; and (E) ELISA results for the *E. coli*-expressed S1 antigen using the SARS-CoV-2 spike S1 monoclonal antibody, showing absorbance values across serial dilutions of the antigen at 4 µg/mL and 40 µg/mL concentrations. mAb: monoclonal antibody; NC: negative control; RBD: receptor binding domain; S1: Spike 1 protein; S2: Spike 2 protein; SP: signal peptide; Try/Trx: Thioredoxin.

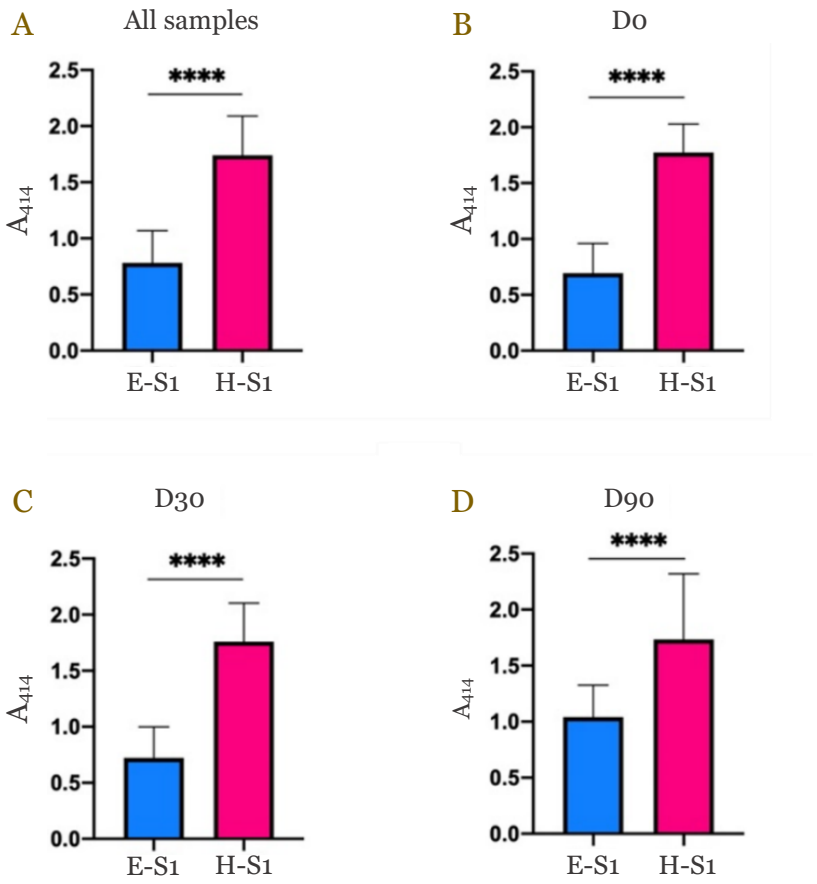

Figure 2. Comparative antibody responses between *Escherichia coli* expression system (E-S1) and HEK293-expressed S1 protein (H-S1) groups. Panels A-D show absorbance at 414 nm in E-S1 (blue) and H-S1 (pink) groups across different time points: (A) All samples combined, (B) Day 0 (D0), (C) Day 30 (D30), and (D) Day 90 (D90). Differences between the two groups were analyzed using the Mann-Whitney U test. Error bars represent the median value with interquartile range (IQR). ****Statistically significant at $p<0.0001$.

Group-specific analysis of correlations between E-S1 and H-S1 antigens

A detailed analysis of the OD values from the E-S1 and H-S1 ELISA was conducted across different participant groups and time points, revealing distinct correlations. Moderate correlations were observed in specific subgroups, indicating that the consistency of antibody detection between E-S1 and H-S1 varied depending on participants' prior exposure to SARS-CoV-2 antigens or the timing of vaccination.

In vaccine recipients with a history of COVID-19 infection, a moderate correlation (r=0.6017; p<0.001) was observed at day 90 post-vaccination (**Figure 3B**). This suggests that in vaccinated individuals previously exposed to SARS-CoV-2, the in-house ELISA using the E-S1 closely aligned with the H-S1 antigen in detecting sustained antibody responses three months post-vaccination. This could be attributed to a more stable or mature immune response that developed over time, resulting in consistent antibody binding across both antigens.

Furthermore, in the naïve vaccine recipients group (those without prior COVID-19 infection), a moderate correlation (r=0.5361; p=0.0003) was observed at day 30 post-vaccination (**Figure 3C**). This finding implies that the E-S1 ELISA can reliably detect early-stage antibody responses in newly vaccinated individuals, providing comparable results to the H-S1-based ELISA within the first month post-vaccination.

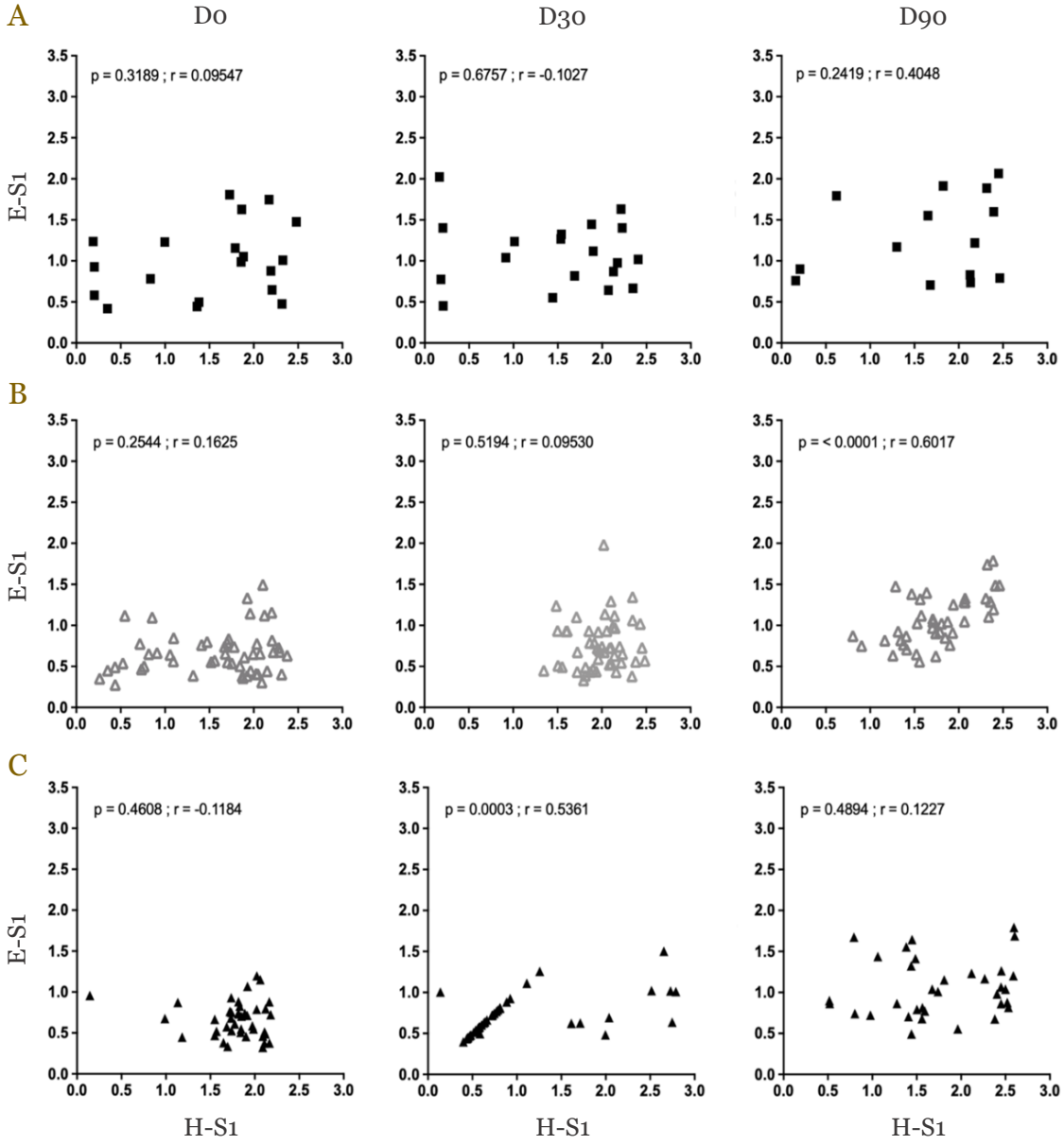

Figure 3. Breakdown analysis of optical density (OD) relationship between *Escherichia coli* expression system (E-S1) and HEK293-expressed S1 protein (H-S1) ELISA. Scatter plots show the Spearman correlation test between E-S1 and H-S1 ELISA OD values across different participant groups at three-time points: Day 0 (D0), Day 30 (D30), and Day 90 (D90). (A) Convalescent (survivor) group; (B) vaccine recipients with a history of COVID-19 infection; and (C) naïve vaccine recipients. The Spearman correlation coefficient (*r*) and *p*-value are indicated for each analysis.

Seropositivity cut-off with pre-pandemic controls

The result showed variability reflecting the timely immune response differences associated with vaccination or infection with significantly higher OD values obtained from H-S1 ELISA in the majority groups at days 0, 30, and 90. At Day 0 (**Figure 4A**), group a (convalescent) exhibited moderate antibody levels, with median OD values of 0.958 (IQR: 0.638–1.195) for E-S1 and 1.807 (IQR: 0.790–2.176) for H-S1 (p=0.0009). Similarly, group b (vaccinated with prior infection) showed medians of 0.641 (IQR: 0.451–0.775) for E-S1 and 1.752 (IQR: 1.057–2.046) for H-S1 (p<0.0001). Group c (vaccinated naive) also demonstrated high antibody levels, with medians of 0.6770 (IQR: 0.498–0.792) for E-S1 and 1.842 (IQR: 1.707–2.042) for H-S1 (p<0.0001). Group d (infection post-vaccination) exhibited variability in antibody levels, with medians of 0.829 (IQR: 0.531–1.257) for E-S1 and 1.221 (IQR: 0.758–1.825) for H-S1, with p=0.0072.

As expected, group e (pre-pandemic controls) had the lowest OD values, with medians of 0.3245 (IQR: 0.228–0.480) in H-S1 ELISA, significantly different from other groups, confirming their seronegative status. Conversely, no significant differences between group e (median: 1.059; IQR: 0.906–1.310) and other groups were observed in E-S1, indicating E-S1 lacks the capacity to differentiate COVID-19 seronegative status with seropositivity derived from natural infection or vaccination. This contrast suggests that the commercial H-S1 protein provides a more reliable threshold for identifying antibody responses post-infection or vaccination due to its greater specificity.

On day 30 and day 90 (**Figure 4B** and **4C**), group a showed moderate antibody levels, with no statistically significant difference between OD values of E-S1 ELISA (day 30 median: 1.040, IQR: 0.776–1.402; day 90 median: 1.195, IQR: 0.783–1.816) and those of H-S1 ELISA (day 30 median: 1.689, IQR: 0.914–2.173; day 90 median: 1.976, IQR: 1.131–2.336). This lack of difference may be due to some non-responders in the H-S1 ELISA who exhibited higher OD values in the E-S1 ELISA, demonstrating the lower specificity of the E-S1.

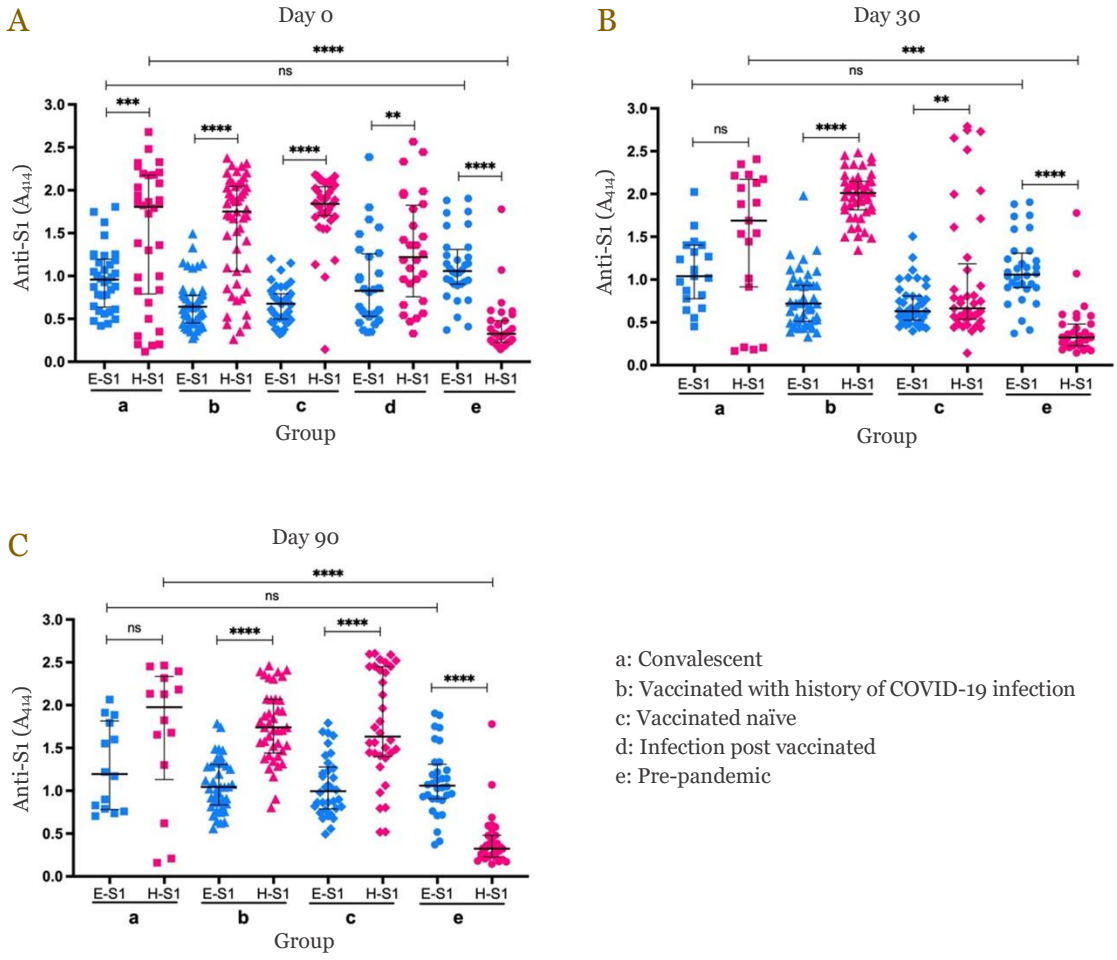

Figure 4. Comparative analysis of seropositivity against *Escherichia coli* expression system (E-S1) and HEK293-expressed S1 protein (H-S1) across different participant groups: convalescent (group a), vaccinated with a history of COVID-19 infection (group b), vaccinated naïve (group c), infection post-vaccination (group d), and pre-pandemic controls (group e). Anti-S1 antibody levels were measured at (A) Day 0, (B) Day 30, and (C) Day 90. Differences between groups were analyzed using the Wilcoxon matched-pairs signed rank test (for groups a, b, c, or d) or the Mann-Whitney test (for groups a vs. e). Significant differences between groups and proteins are indicated as ns (not significant); $^{**}p<0.01$; $^{***}p<0.001$; $^{****}p<0.0001$.

Specificity assessment with pre-pandemic sera

Pre-pandemic sera from Sumba Island were further assessed to investigate the specificity of the E-S1 ELISA and to determine whether elevated OD values were due to cross-reactivity (**Figure 5**). To this end, we conducted E-S1 ELISA and H-S1 ELISA on several pre-pandemic sera from Jakarta City and compared the results with those of Sumba samples. Since the H-S1 ELISA of Sumba samples consistently showed low OD values, the high absorbance readings observed with E-S1 were unlikely due to SARS-CoV-2 cross-reactivity (**Figure 5A**). This observation suggests that the elevated signals with E-S1 may stem from background noise, possibly due to anti-*E. coli* antibodies in the pre-pandemic sera. Consequently, while the E-S1 antigen retains relevant antigenic epitopes, the presence of non-specific reactivity with pre-existing antibodies poses a limitation in using the *E. coli*-expressed spike protein as a COVID-19-specific ELISA antigen. In contrast, pre-pandemic sera collected from a more urban and educated population in Jakarta City, the capital of Indonesia, showed a strong correlation between E-S1 and H-S1 ELISA OD values, indicating a higher likelihood of shared epitopes between both (**Figure 5B**).

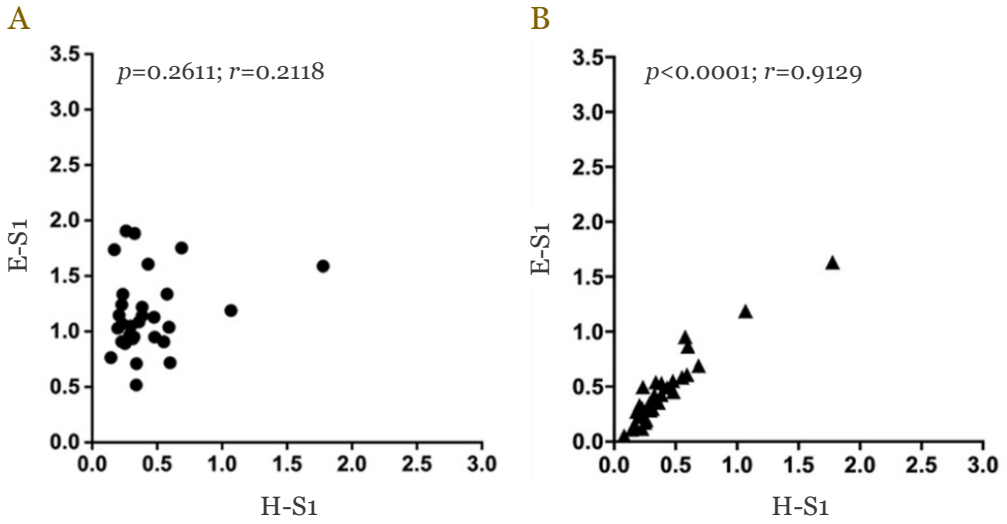

Figure 5. Correlation of optical densities (ODs) between *Escherichia coli* expression system (E-S1) and HEK293-expressed S1 protein (H-S1) ELISA from the pre-pandemic sera obtained from (A) Sumba Island and (B) DKI Jakarta province. The Spearman correlation coefficient (*r*) and *p*-value are indicated for each analysis.

Discussion

The results demonstrate the potential of E-S1 for serological testing but also reveal significant limitations when compared to H-S1. These differences illuminate the critical factors affecting the diagnostic performance of these antigens, including their structural integrity, antigenic properties, and the immune backgrounds of tested populations.

H-S1 consistently exhibited superior sensitivity and specificity across all groups and time points. This advantage is likely due to the preservation of native conformational epitopes essential for efficient antibody recognition and binding. Mammalian cell-expressed antigens, such as H-S1, undergo proper folding and post-translational modifications, such as glycosylation, which are crucial for maintaining the structural and functional integrity of the spike protein. A previous study has highlighted that human cell-expressed SARS-CoV-2 antigens exhibit robust sensitivity and specificity due to their ability to mimic the native viral structure, making them highly effective for serological assays [30]. Conversely, *E. coli*-expressed antigens lack glycosylation and are prone to misfolding, leading to reduced antigenicity. Similar findings were reported that bacterial-expressed proteins often suffer from structural limitations, which negatively impact their diagnostic utility [31]. Our study corroborates these observations, as E-S1 demonstrated reduced sensitivity and specificity, particularly when distinguishing convalescent individuals from seronegative controls.

The variability in E-S1 performance was further evident in group-specific correlation analyses. Moderate correlations between E-S1 and H-S1 were observed at certain times, particularly in vaccinated individuals with prior COVID-19 infection and in naïve vaccine recipients shortly after vaccination. These findings suggest that E-S1 may detect antibody responses in specific contexts, such as late post-vaccination or individuals with mature immune responses due to prior SARS-CoV-2 exposure. Similarly, a study has shown variable performance in serological assays depending on participants' antigen source and immune background, emphasizing the influence of individual immune history on assay outcomes [32]. However, the inconsistent correlations across other groups in our study highlight the limited utility of E-S1 compared to H-S1. This aligns with the previous findings that showed mammalian-expressed spike proteins consistently provide better diagnostic accuracy across diverse participant groups, regardless of prior exposure or vaccination status [33].

The specificity assessment using pre-pandemic sera provided additional insights into the challenges posed by E-S1. Elevated OD values in sera from a rural population in Sumba Island indicate possible non-specific reactivity, likely due to anti-*E. coli* antibodies or other cross-reactive immune responses to bacterial antigens. These results align with the previous findings, which reported high non-specific reactivity in assays using bacterial-expressed antigens, particularly in populations with frequent bacterial exposures [34,35]. Similarly, bacterial expression systems often introduce contaminating proteins or shared epitopes that can lead to cross-reactivity in serological assays, potentially influenced by regional or environmental factors [15,36]. Interestingly, pre-pandemic sera from a more urban and educated population in Jakarta City showed a strong correlation between E-S1 and H-S1, potentially reflecting differences in environmental exposures and immune priming, or pre-existing antibodies, such as those generated against bacterial antigens. Urban populations often exhibit distinct antibody patterns compared to rural populations due to differences in pathogen exposure and healthcare access [37,38].

Overall, these results emphasize the limitations of E-S1 as a COVID-19-specific antigen due to its reduced sensitivity, non-specific reactivity, and variability across populations. The challenges associated with *E. coli* expression systems, particularly the lack of glycosylation and improper folding, likely contribute to these shortcomings. Future studies should optimize E-S1 production to minimize non-specific reactivity and enhance structural integrity to improve its diagnostic performance. Alternatively, switching to mammalian or yeast expression systems could preserve conformational epitopes and reduce cross-reactivity, making the antigen more suitable for broad serological applications.

Conclusion

E-S1 shows some promise for serological testing; however, its diagnostic performance is limited compared to the more robust and reliable H-S1 antigen. These findings highlight the critical role of antigen source and structure in determining assay sensitivity and specificity, emphasizing the importance of tailoring diagnostic tools to address population-specific immune profiles. Further studies reinforce the need for antigen optimization to enhance the accuracy and reliability of serological platforms for SARS-CoV-2 and other emerging infectious diseases.

Ethics approval

The Hasanuddin University Faculty of Medicine's Institutional Ethics Committee has approved this study (4/UN4.6.4.5.31/PP36/2021).

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

Declaration of artificial intelligence use

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

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