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Synthetic peptide-based latex agglutination test for the detection of *Bacillus anthracis* spores from soil samples

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The environmental persistence of *Bacillus anthracis* spores continues to challenge rapid detection and surveillance of anthrax, necessitating simple, field-deployable analytical tools. This study aimed to develop a peptide-directed IgY-based latex agglutination test (LAT) as a rapid, cost-effective analytical method for the selective detection of *B. anthracis* spores. Four species-specific peptides derived from protective antigen (PA) and S-layer (EA1) proteins were identified through bioinformatic screening and used to generate polyclonal IgY antibodies. Latex beads (1.25%) were functionalized with optimized antibody concentrations to develop the LAT. Analytical performance was evaluated in terms of sensitivity, specificity, and cross-reactivity using inactivated spores of *B. anthracis* and related *Bacillus* spp., with validation by indirect ELISA. Applicability in complex matrices was assessed through spiking studies in soil and meat meal samples, followed by evaluation using 257 field samples from anthrax-endemic regions, benchmarked against WOAH-recommended *pag* gene PCR. The optimized LAT (200 μg ml^{-1} IgY) achieved a detection limit of 10^5 spores per ml, with high specificity for peptides PA-1 and EA-1 and minimal cross-reactivity. ELISA corroborated the specificity of peptide-derived IgY antibodies. In spiked matrices, recovery efficiency ranged from 50–75%, with a practical detection limit of 10^6 spores per g. Field validation demonstrated 72.7% sensitivity and 100% specificity ($\kappa = 0.83$) relative to PCR. This study presents a novel peptide-based immunoanalytical platform for on-site biosurveillance and resource-limited analytical settings that integrates specificity, rapid response, and low cost, offering a practical alternative to conventional methods for environmental monitoring of *B. anthracis*.

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1 Introduction

Anthrax, a zoonotic disease caused by *Bacillus anthracis*, persists as a global public health concern.[1] The bacterium exists as vegetative cells in hosts and as highly resistant spores in the environment, enabling long-term soil survival and transmission among herbivores and livestock.[2] In humans, anthrax manifests as inhalational, cutaneous, or gastrointestinal forms, often progressing to fatal systemic infection.[3] Despite reduced global incidence, sporadic re-emergence, including intentional outbreaks, highlights its ongoing threat.[4] Historically, 20 000–100 000 cases occurred annually (Morris, 1999),[5] with the World Health Organization (WHO) now estimating 2000–20 000 cases per year.[6] Approximately 1.1 billion animals and 1.83 billion humans are at risk of exposure to anthrax spores from soil and the environment, particularly in Africa, Asia, and Europe.[7] The disease is endemic in several regions, including India, where it ranks among the top 10 livestock diseases.[8] The WHO Collaborating Centre for Geographic Information Systems and Remote Sensing for Public Health has recorded animal outbreaks in nearly 200 countries.[9]

The persistence of the spores in the environment, especially in the soil, is considered a key factor for their endemicity and disease transmission in herbivores and domestic animals. Generally, *B. anthracis* spores are formed by the bacterium when environmental conditions become unfavorable for its growth,

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thus allowing them to survive in a metabolically inactive state for an extended period. These *B. anthracis* spores can persist for decades in calcium-rich soil with a pH above 6.0 and temperatures above 15.5 °C.[10] High-risk groups include farmers, butchers, and veterinarians, with cutaneous anthrax constituting over 95% of cases.[11] Economically, anthrax causes severe losses in the meat, leather, and wool sectors despite vaccination programs.[9]

Detection of *B. anthracis* in clinical and environmental samples remains challenging. The conventional culture method, though the gold standard for identifying vegetative cells, is labour-intensive, requires biosafety level (BSL)-3 facilities, and takes 1–2 days to yield results.[12] Advanced nucleic acid-based assays,[13] and protein profiling techniques such as microfluidic capillary electrophoresis offer greater sensitivity and faster detection,[13] but their use is restricted by the need for costly infrastructure and technical expertise.[14] Immunoassays targeting *B. anthracis* vegetative cells, spores, or toxins[15] have been developed, yet antigenic cross-reactivity with other *Bacillus* spp. remains a limitation.[16] Comparative whole-genome sequencing reveals 91.4–100% identity among *Bacillus* isolates, complicating differentiation by conventional means.[17] Although WOAH, BIS, and WHO (2008)[18] protocols recommend culture-based confirmation, these are time-consuming, resource-intensive, and unsuitable for rapid field diagnosis. Latex agglutination test (LAT) could serve as an effective alternative screening method, as it has been proven to be a rapid diagnostic tool for the detection of several important infectious diseases, such as brucellosis,[19] streptococcosis,[20] tularemia,[21] and *E. coli* infections.[22]

In view of these constraints, this study aims to develop a LAT employing specific synthetic peptides derived polyclonal antibodies for rapid, reliable, and safe detection of *B. anthracis* spores in animal feed and soil samples. The LAT assay is simple, rapid, and non-hazardous,[23] with over 400 documented research articles demonstrating its utility across more than 300 diseases and biomolecules in veterinary medicine and related fields.[24] In India, limited studies have explored LAT for the detection of pathogenic *B. anthracis*. Previous research documented protective antigen (PA)-based peptides for testing of clinical samples, *i.e.*, blood or serum.[25,26] Building on our earlier study employing characterized PA-based peptides for anthrax spore detection,[6] the present research represents the first attempt of its kind to develop a synthetic-peptide-derived antibody-based LAT assay for the detection of *B. anthracis* spores in animal feed supplements and soil samples. Overall, this study addresses a critical diagnostic gap by providing a rapid, field-adaptable, and cost-effective screening tool capable of distinguishing *B. anthracis* from closely related *Bacillus* species from soil and animal feed supplements.

2 Experimental

2.1 Bacterial strains

Standard strains of *Bacillus* spp. (*B. thuringiensis* VTCCBAA1527, *B. cereus* VTCCBAA1538, *B. licheniformis* VTCCBAA1055, and *B. mycoides* VTCCBAA479) were procured from the National Centre for Veterinary Type Culture Collection, ICAR-National Research Centre on Equines, Hisar, India.

The *B. anthracis* Sterne strain (34F2) was revived from the live attenuated anthrax spore vaccine (IAHVB, Bengaluru) by incubating spores in Luria–Bertani (LB) broth.[27] Vegetative cells were obtained by heat activation of the avirulent spore vaccine at 70 °C for 15–30 min in LB broth, followed by streaking on selective polymyxin-lysozyme-EDTA-thallous acetate (PLET) agar and 5% sheep blood agar to confirm characteristic colony morphology and non-hemolytic patterns, respectively.[28] Gram staining revealed the typical bamboo stick or boxcar-shaped morphology, and PCR targeting the WOAH-recommended *B. anthracis* pXO1 plasmid-encoded *pag* gene confirmed the identity of the heat-activated Sterne strain.[6,29]

Additionally, all the *Bacillus* reference cultures and the *B. anthracis* Sterne strain were biochemically characterized using the Automatic Identification System with the Vitek-2 Compact analyzer (bioMérieux). Approval for handling these pathogens was obtained from the Institutional Biosafety Committee.

2.2 Chemicals

Reagents for development of the LAT, including Polybead® Carboxylate Red polystyrene dyed microspheres (1.10 μm, cat#19120-15) and Polybead® Carboxylate Blue polystyrene dyed microspheres (1.00 μm, cat#19119-15), were procured from Polysciences Inc., USA.

Chemicals required for conjugating latex beads with specific antibodies, namely MES buffer (2-morpholinoethane sulfonic acid hydrate; cat#145224-94-8, 100 g) and EDAC buffer (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; cat#E-2247), were obtained from Sisco Research Laboratories (SRL) Pvt. Ltd, India, and Thermofisher Scientific, USA, respectively.

2.3 Designing and synthesizing *B. anthracis*-specific synthetic peptides

Bacillus anthracis-specific peptides were identified by retrieving the sequences of the Protective Antigen (PA) protein (NCBI Protein ID: AAT28905) and EA1 (S-layer transmembrane) protein (NCBI Protein ID: AAT53169) of the *B. anthracis* Ames strain from the NCBI database. The retrieved sequences were analyzed for transmembrane regions using TOPCONS (https://topcons.net) and the IEDB analysis resource. Identified regions were further evaluated for antigenicity (B-cell epitopes), surface probability, hydrophilicity, turn, and flexibility using the Protean module of LASERGENE software. The highly specific peptide sequences identified for the detection of *B. anthracis* (NCBI protein BLAST), *i.e.*, four specific peptides-two each from PA and EA1 proteins-were selected for synthesis and diagnostic evaluation. All selected peptides (Table 1) were synthesized at ≥90% purity by high-performance liquid chromatography (HPLC) through S BioChem, GMR Foundations, Kerala.

2.4 Spore preparation and inactivation

Spores of *B. anthracis* Sterne strain (34F2) and related *Bacillus* spp. were prepared by seeding heat-activated *B. anthracis*

Table 1 Details of peptides used in this study

S. no.	Peptide code	Sequence	Amino acid (no.)	Orientation	Purity	Molecular weight (daltons)
1	BA-PA-1	IYNVLPTTSLVLGK-amidated	14	Linear	≥90%	1517.96
2	BA-PA-2	LNLVERRIAAVNPS-amidated	14	Linear	≥90%	1551.91
3	BA-EA-1	VTKFVYEVKKLAVEKL-amidated	16	Linear	≥90%	1894.55
4	BA-EA-2	KLQVVQNKTLPVTFV-amidated	15	Linear	≥90%	1714.28

vaccine strain on PLET medium, followed by incubation at 37 °C for 5 days until ≥95% sporulation.[6,28] Sporulation was confirmed microscopically using the Schaeffer–Fulton spore staining method.[30] Spores were harvested in 1× PBS with 1% BSA, serially diluted, and plated on PLET agar for enumeration.[31]

For inactivation, harvested spores (10^{10} spores per ml) were subjected to UV irradiation, moist heat (70 °C and 100 °C in a water bath), or dry heat (70 °C and 100 °C in a hot-air oven) at 10, 30, 60, and 120 minutes.[6,32] Similarly, soil samples (7.5 g) spiked with 10^{10} spores per ml were treated under identical conditions to simulate environmental settings. Viability and antigenicity of inactivated spores were assessed by plating on PLET agar and testing with the optimized LAT.[6] Confirmed inactivated spores and controls were stored at room temperature, 4 °C, 0 °C, and −20 °C and evaluated weekly for antigenicity. Further, the effect of UV inactivation on DNA quality was assessed by PCR employing the *pag* gene[6,44] using DNA extracted from both UV-inactivated and live spores. All procedures were conducted aseptically in a BSL-3 Cabinet (IMSET, Mumbai, India).

2.5 Preparation of IgY antibodies

The polyclonal IgY antibodies against each synthesized peptide were generated by outsourcing the synthesized peptides (5 mg) to IgY Immunologix Pvt. Ltd, Hyderabad.

2.6 Determination of the specificity of peptide-derived antibodies by indirect ELISA

An indirect ELISA was employed to assess the specificity of IgY antibodies raised against the selected peptides (PA1, PA2, EA1, and EA2) using UV-inactivated spores of related *Bacillus* species (*B. anthracis*, *B. cereus*, *B. licheniformis*, *B. thuringiensis*, and *B. mycoides*), as described by Liu *et al.* in 2016 (ref. 33) with suitable modifications.

Briefly, 10^4 UV-inactivated spores of each *Bacillus* species were suspended in carbonate-bicarbonate buffer (pH 9.6), and 100 µl of each suspension was placed in ELISA plate wells in triplicate. Plates were incubated at 37 °C for 1 h, followed by overnight incubation at 4 °C. After three washes with washing buffer (300 µl per well), plates were blocked with 300 µl of blocking buffer (5% skimmed milk and 1% BSA) at 37 °C for 2 h.

Serial dilutions of primary IgY antibodies (1 : 50, 1 : 100, 1 : 200, *etc.*) in PBS were added (100 µl per well) and incubated at 37 °C for 1 h. After washing, wells were treated with HRP-conjugated anti-chicken IgY (1 : 5000 dilution) for 1 h at 37 °C. Colour development was achieved using 100 µl of substrate solution [10 mg *o*-phenylenediamine dihydrochloride (OPD) in 10 ml phosphate-citrate buffer (pH 5.0) containing 6 µl H_2O_2] and incubating in the dark for 15 min. The reaction was stopped with 50 µl of 1 M H_2SO_4, and absorbance was measured at 492 nm using an ELISA reader (LisaScan EM, Transasia Bio-Medicals Ltd, India). The positive cut-off value was calculated using:

$$\text{Cut-off value} = (\text{mean OD of negative control}) + 2 \times \text{standard deviation}$$

2.7 Preparation of LAT reagent and optimization of LAT assay

LAT reagents were prepared using 1.25% blue-dyed carboxylate latex beads with IgY antibodies against identified peptides at concentrations of 100, 200, and 250 µg ml^{-1}.[6,34,35] Conjugation of latex beads with peptide-specific antibodies was performed *via* covalent coupling.[6,34–37] The test was optimized by checkerboard titration using varying reagent volumes and known positive and negative inactivated spores in a BSL-3 cabinet. For testing, 10 µl of conjugated reagent was mixed with an equal volume of test spores in a concavity well, rotated for 1 min, and observed. Positive samples showed visible agglutination within 2 min, while negative controls showed none.

2.8 Determining the sensitivity and specificity of LAT assays

The LAT reagents prepared with optimized concentrations of IgY antibodies against the selected peptides (PA-1, PA-2, EA-1, and EA-2) were evaluated for sensitivity using serial dilutions of UV-inactivated *B. anthracis* spores (10^6, 10^5, 10^4, 10^3, 10^2, 10 spores per ml). Specificity was assessed against inactivated spores of related *Bacillus* spp. (*B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. subtilis*, and *B. licheniformis*) and other pathogenic Firmicutes (*Staphylococcus aureus* and *Listeria monocytogenes*). To simulate natural environmental conditions, spore mixtures (10^{10} spores per ml) with varying proportions of *B. anthracis* and related *Bacillus* spp. (100 : 0, 75 : 25, 50 : 50, 25 : 75, 0 : 100) were tested with the optimized LAT reagents. Among the four peptides tested, PA-1 and EA-1 demonstrated 100% specificity against *B. anthracis* spores with no cross-reactivity and were therefore selected for further spiking studies and field sample screening for comparative evaluation with the WOAH-recommended test.

2.9 Preparation of sterile soil/meat meal samples for spiking studies

Sterile soil and meat meal samples were prepared by moist and dry heat sterilization[38] for artificial spiking with spores of *B.*

anthracis and related *Bacillus* spp. to evaluate the developed LAT for sensitivity and specificity. The spiked spores were separated using the Ground Anthrax Bacilli Refined Isolation (GABRI) method.

In brief, alkaline soil (pH 8.5) was sieved, repeatedly washed with distilled water, spread in Petri dishes, and pre-incubated at 37 °C for 2 days to induce spore germination. Sterilization was performed by autoclaving (121 °C, 15 lbs, 1 h) and by dry heat (200 °C, 24 h); the moist heat cycle was repeated to ensure complete spore destruction. Sterility was confirmed by incubation in BHI/nutrient broth at 37 °C for 24 h, followed by streaking on PLET agar. A similar method was adopted for meat meal samples procured from the market. Sterile samples were stored in screw-capped bottles for subsequent assays.

2.10 Recovery of spiked *Bacillus* spores from soil/meat meal employing the GABRI method

The GABRI method was performed to recover *B. anthracis* spores from spiked soil or meat meal samples as described previously,[39] with suitable modifications. In brief, five aqueous carrier media-Triton™ X-100 in deionized water, Tween-20 in PBS, sucrose solution, Triton™ X-100 in sucrose, and Tween-20 in sucrose-were evaluated for their efficiency in separating *B. anthracis* spores from soil.[40–42]

Briefly, 22.5 ml of each carrier medium was added to 7.5 g of spiked (10^{10} spores) soil or meat meal sample and vortexed for 30 min. The suspension was centrifuged at 2000 rpm for 5 min to remove gross debris. The resulting supernatant was concentrated and analyzed for spore count and agglutination with the LAT. The best-performing carrier medium was selected based on the spore recovery rate, calculated as:

$$\text{Spore recovery rate} = \frac{\text{Recovered spore count}}{\text{Spiked spore count}} \times 100$$

2.11 Sensitivity and specificity of LAT in spiked soil/meat meal

The sensitivity of the optimized LAT was determined in artificially spiked sterile soil and meat meal samples as described earlier,[43] with suitable modifications. Samples (7.5 g) were spiked with varying concentrations of live *B. anthracis* spores (10^{10}, 10^{8}, 10^{6}, 10^{4}, and 10^{2}) and recovered using the GABRI technique with sucrose-Tween 20 as the carrier medium. Recovered spores were enumerated on PLET agar by serial dilution, and the UV-inactivated spores were tested for agglutination using optimized LAT assays.

The specificity of the LAT was assessed similarly,[6,43] by spiking sterile soil and meat meal samples (7.5 g) with spores of *B. anthracis* and closely related *Bacillus* spp. (*B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. licheniformis*). Spore recovery was performed using the GABRI technique with sucrose-Tween 20 as the carrier medium. Recovered live spores were counted on PLET agar, while UV-inactivated spores were analyzed by optimized LAT assay.

Further, to simulate environmental conditions and ensure the specificity of the LAT assay, mixed spore suspensions (10^{10} ml^{-1}) containing *B. anthracis* and related *Bacillus* spp. in various proportions (100 : 0, 75 : 25, 50 : 50, 25 : 75, and 0 : 100) were prepared and spiked into sterile soil and meat meal samples. The spores were harvested as stated above and then evaluated to assess cross-reactivity and specificity using the optimized LAT assays.

2.12 Collection and processing of soil and animal feed supplement samples for evaluation of optimized LAT assay with WOAH-recommended PCR assay

A total of 257 field samples (soil and feed supplements) were collected from anthrax-endemic and outbreak-prone regions of India using a simple random sampling method without replacement (Table 5).

Approximately 50–100 g of each sample was collected following appropriate biosafety protocols, including the use of full personal protective equipment and disinfection with 5–10% sodium hypochlorite (10 000 ppm). Samples were UV-inactivated on-site using a portable UV sterilizer, double-packed in biohazard-labelled containers, and transported under safe conditions to the laboratory.

Spore separation was performed using the GABRI method. The recovered spores were divided into two equal parts; one part was used to extract DNA using the Power Soil DNA Isolation Kit (Qiagen Cat# 12888), and the obtained DNA was used to detect *B. anthracis* spores employing the WOAH-recommended PCR assay,[6,29] while the remaining part was used to test *B. anthracis* spores by the optimized LAT assay. PCR primers (Eurofins India) targeting the *B. anthracis* pXO1 plasmid-encoded protective antigen (*pag*) gene[6,44] are listed in SI Table S1.

All field sample processing was performed in a Class III biosafety cabinet (ImSet, Mumbai, India). Contaminated disposables (falcon tubes, pipette tips, tissues, buffers) were autoclaved and discarded following institutional biosafety protocols. Diagnostic sensitivity and specificity of the LAT were calculated relative to the WOAH-recommended PCR assay, and statistical analyses were performed to assess overall assay performance.

2.13 Statistical analysis

Statistical analyses, including Cohen's kappa for agreement for diagnostic performance, were performed to evaluate the overall reliability of the developed LAT assays.

3 Results and discussion

Anthrax, caused by *Bacillus anthracis*, is a deadly zoonosis affecting humans and livestock, especially cattle and sheep, with significant economic impact.[45] Its persistence as resistant spores in soil contributes to recurrent outbreaks, particularly in endemic regions of India and other parts of Asia, Africa, and Europe.[8] Conventional culture and molecular detection methods are time-consuming, infrastructure-intensive, and often limited by cross-reactivity with related *Bacillus* species.[12]

Table 2 Spore inactivation of *B. anthracis* and corresponding spore count reduction following exposure to various physical methods

			Direct exposure to spores			Exposure to spores spiked in soil		
Method of inactivation	Temperature/UV	Time (min)	Viability	Antigenicity	Log unit (log_{10} CFU ml^{-1})	Viability	Antigenicity	Log unit (log_{10} CFU g^{-1})
Moist heat method (water bath)	70 °C	10	Viable	Retained	8.78	Viable	Retained	9.84
		30	Viable	Retained	3.95	Viable	Retained	4.90
		60	Inactivated	Lost	0	Inactivated	Lost	0
		90	Inactivated	Lost	0	Inactivated	Lost	0
		120	Inactivated	Lost	0	Inactivated	Lost	0
	90 °C	10	Viable	Retained	5.30	Viable	Retained	6.70
		30	Viable	Retained	2.00	Viable	Retained	2.78
		60	Inactivated	Lost	0	Inactivated	Lost	0
		90	Inactivated	Lost	0	Inactivated	Lost	0
		120	Inactivated	Lost	0	Inactivated	Lost	0
Dry heat method (hot air oven)	70 °C	10	Viable	Retained	9.78	Viable	Retained	9.90
		30	Viable	Retained	4.95	Viable	Retained	6.00
		60	Inactivated	Lost	0	Inactivated	Lost	0
		90	Inactivated	Lost	0	Inactivated	Lost	0
		120	Inactivated	Lost	0	Inactivated	Lost	0
	90 °C	10	Viable	Retained	5.95	Viable	Retained	6.95
		30	Viable	Retained	3.30	Viable	Retained	3.70
		60	Inactivated	Lost	0	Inactivated	Lost	0
		90	Inactivated	Lost	0	Inactivated	Lost	0
		120	Inactivated	Lost	0	Inactivated	Lost	0
UV-inactivation	30 watts UV light, 2 feet distance	10	Viable	Retained	8.90	Viable	Retained	9.60
		30	Viable	Retained	3.08	Viable	Retained	6.78
		60	Inactivated	Retained	0	Viable	Retained	2.00
		90	Inactivated	Retained	0	Inactivated	Retained	0
		120	Inactivated	Retained	0	Inactivated	Retained	0

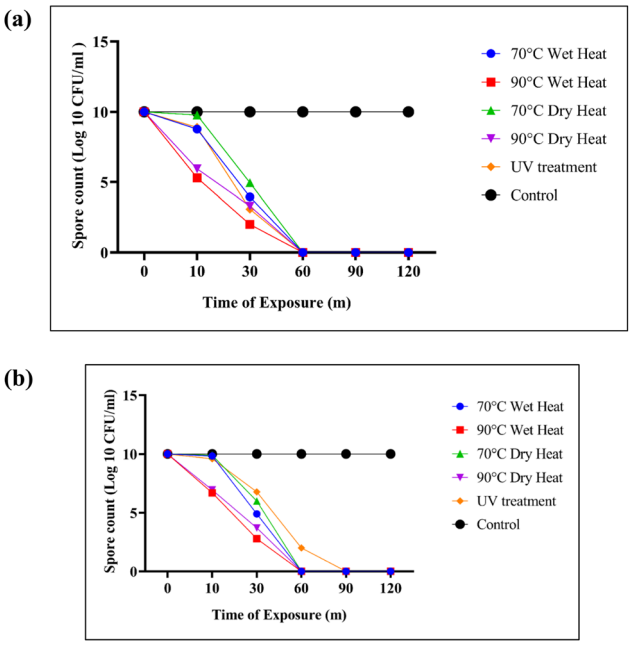

Fig. 1 *B. anthracis* spore count (log_{10} CFU ml^{-1}) after inactivation with different methods. Image (a) represents plain spores, while image (b) denotes spiked samples.

To address these limitations, this study developed and evaluated a latex agglutination test (LAT) using *B. anthracis*-specific synthetic peptides and their derived polyclonal IgY antibodies, aiming to provide a rapid, specific, and cost-effective tool for discriminating *B. anthracis* spores from closely related species in routine laboratory and field settings.

3.1 Spore preparation and inactivation

Sporulation of *B. anthracis* Sterne (34F2) and other *Bacillus* species became evident by day 3 and exceeded 95% by day 6 for most species, except *B. mycoides*, which sporulated by day 5 (SI Fig. S1). Microscopic examination using Schaeffer–Fulton staining revealed characteristic green spores within pink bacilli (SI Fig. S2a and b). Similar sporulation timelines have been reported previously, where optimal sporulation of *B. anthracis* occurs within 3–6 days under suitable growth conditions, influenced by factors such as strain type, temperature, and nutrient limitation.[46–48]

Evaluation of different physical inactivation methods showed that complete spore inactivation was achieved after 1 h of both moist and dry heat treatment; however, these treatments resulted in loss of antigenicity (Table 2). In contrast, UV irradiation achieved complete inactivation while preserving antigenic integrity, with effective exposure times of 1 h for plain spores and 1 h 30 min for soil-spiked spores (Fig. 1). The higher susceptibility of spores in aqueous suspension compared with dried spores has been attributed to differences in surface characteristics and UV penetration, while spores are generally reported to be 5–10 times more resistant to UV radiation than vegetative cells.[49,50] Differences in reported UV inactivation

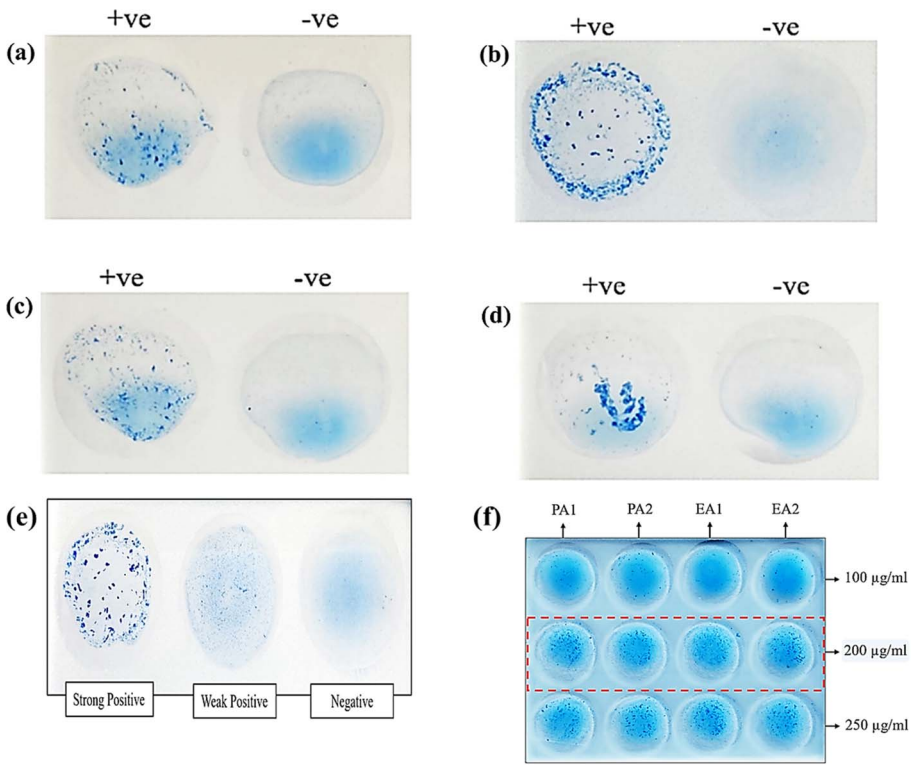

Fig. 2 LAT assay with known positive and negative controls. Images (a) denote PA1 peptide-based LAT, (b) represent PA2 peptide-based LAT, (c) illustrate EA1 peptide-based LAT, (d) represent EA2 peptide-based LAT, (e) denote LAT with strong positive, weak positive, and negative control, and (f) represent optimization of LAT with different concentrations of IgY antibodies. +ve: *B. anthracis* spore (Sterne vaccine strain), while −ve: 1× Phosphate Buffered Saline (PBS).

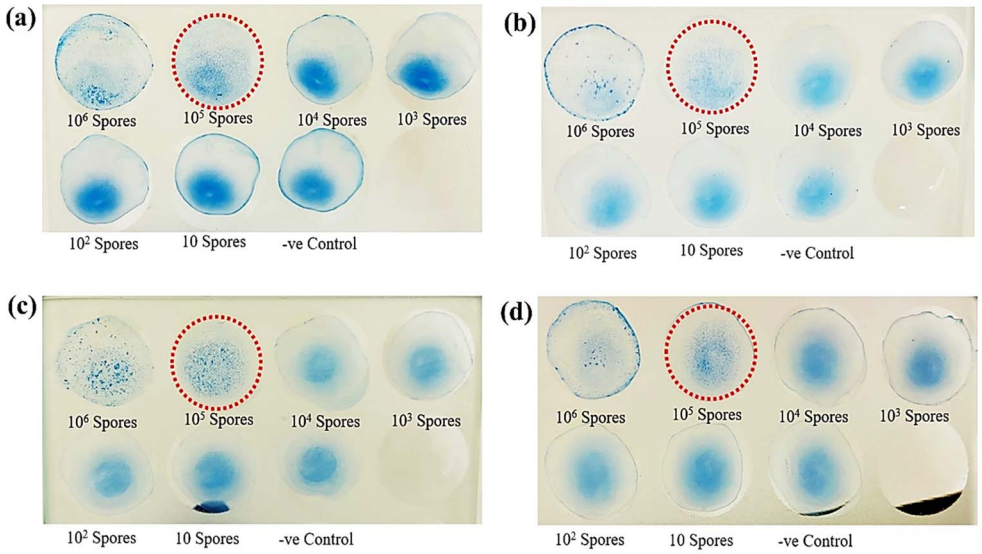

Fig. 3 Sensitivity of the optimized LAT with different concentrations of *B. anthracis* spores. Images (a) PA1 peptide-based LAT, (b) PA2 peptide-based LAT, (c) EA1 peptide-based LAT, and (d) EA2 peptide-based LAT.

efficiencies across studies may arise from variations in strain type, spore concentration, and spore state.[28,51,52] Moreover, no difference could be observed in the PCR amplification pattern between DNA extracted from inactivated and live spores (SI Fig. S3a).

Assessment of antigenic stability during storage revealed that UV-inactivated spores maintained consistent agglutination when stored at −20 °C, whereas a marked decline in antigenicity was observed at higher storage temperatures after two weeks (SI Fig. S3b). These findings indicate that UV irradiation is an effective method for spore inactivation while retaining antigenicity, and that storage at −20 °C preserves antigenic stability for subsequent immunodiagnostic applications.

3.2 Optimization of the LAT assay

LAT reagents prepared with IgY antibodies raised against the synthetic peptides (PA-1, PA-2, EA-1, and EA-2) produced rapid and distinct agglutination with inactivated *B. anthracis* spores (10^6 spores per ml) within 1 min, while no agglutination was observed with the negative control, confirming reagent stability and absence of autoagglutination (Fig. 2a–e). Among the tested antibody concentrations (100, 200, and 250 μg ml^{-1}), 200 μg ml^{-1} yielded clear and consistent agglutination comparable to the other concentrations across all peptide-derived IgY antibodies (Fig. 2f) and was therefore selected as the optimal concentration for LAT reagent preparation.

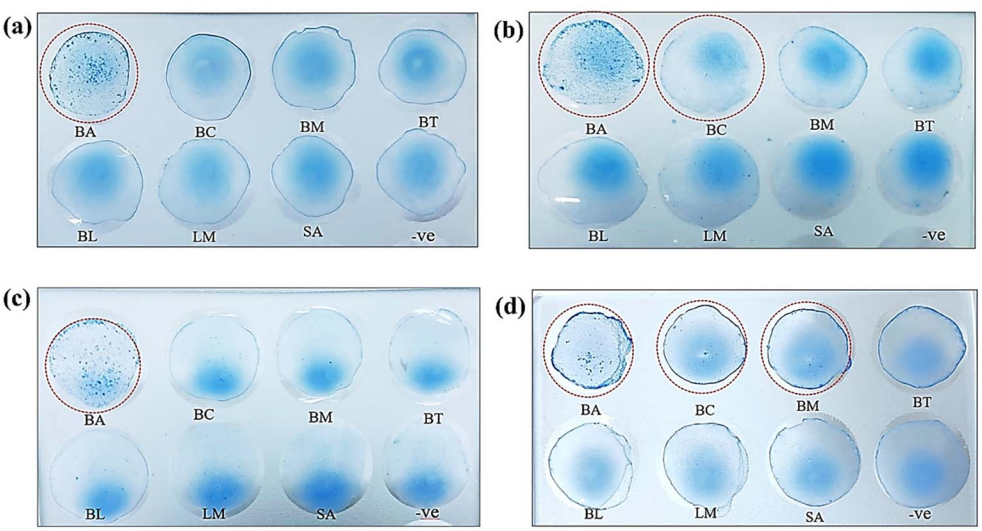

Fig. 4 Specificity of the optimized LAT with the spores of different *Bacillus* spp. Images (a) PA1 peptide-based LAT, (b) PA2 peptide-based LAT, (c) EA1 peptide-based LAT, and (d) EA2 peptide-based LAT. BA: *B. anthracis*, BC: *B. cereus*, BM: *B. mycoides*, BT: *B. thuringiensis*, BL: *B. licheniformis*, LM: *L. monocytogenes*, SA: *S. aureus*, −ve: negative control.

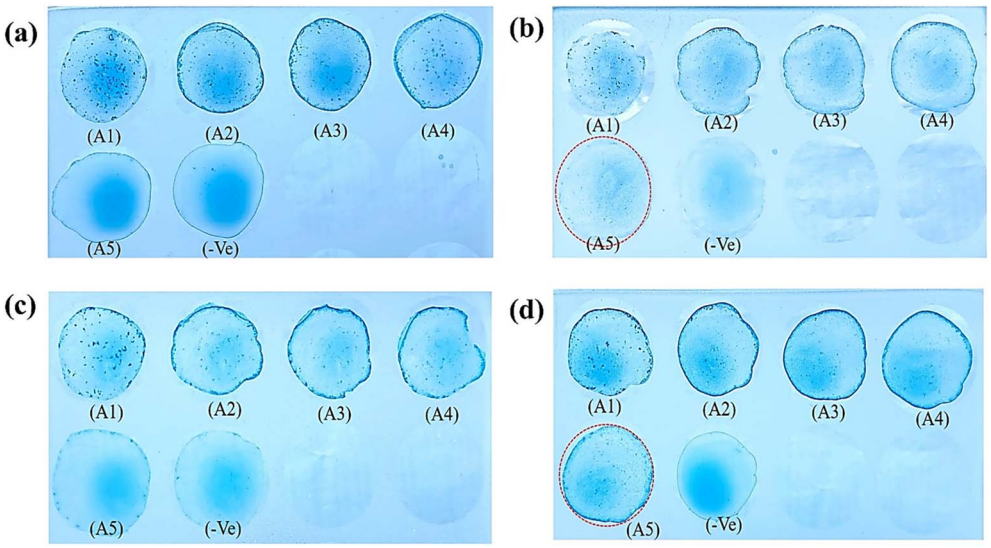

Fig. 5 Specificity of the optimized LAT with the different proportions of the spores of *Bacillus* spp. Images (a) denote PA1 peptide-based LAT, (b) PA2 peptide-based LAT, (c) EA1 peptide-based LAT, and (d) EA2 peptide-based LAT. A1: 100% *B. anthracis*, A2: 75% *B. anthracis* + 25% other *Bacillus* spp, A3: 50% *B. anthracis* + 50% other *Bacillus* spp. A4: 25% *B. anthracis* + 75% other *Bacillus* spp. A5: 100% other *Bacillus* spp. −ve: negative control.

The strong performance of the peptide-based LAT reagents is supported by the high theoretical specificity of the selected *B. anthracis* peptides derived from PA and EA1 proteins (SI Fig. S4–S7). Bioinformatic analyses, including B-cell epitope prediction, surface probability, hydrophilicity, and NCBI BLAST searches, indicated minimal similarity with non-anthrax *Bacillus* species, suggesting a low likelihood of cross-reactivity. Similar observations have been reported for short synthetic peptides that selectively bind *B. anthracis* spores, where specificity is influenced by the presence of key amino acid residues within the epitope sequence.[6,53–55]

The use of IgY antibodies further contributed to the assay performance, as IgY is known to provide advantages over mammalian IgG, including higher antibody titers, broader epitope recognition, improved stability, and lack of interference from mammalian rheumatoid factors.[56–58] The optimal agglutination observed at 200 μg ml^{-1} in the present study is comparable with findings from peptide-based LAT systems reported previously,[6] although optimal concentrations may vary depending on the antibody and antigen used.[25,59] Overall, these results demonstrate that peptide-specific IgY antibodies, when appropriately titrated, provide a reliable and specific platform for LAT-based detection of *B. anthracis* spores.

3.3 Determining the sensitivity and specificity of LAT assays

LAT reagents prepared with the optimized IgY antibody concentration (200 μg ml^{-1}) against the four synthetic peptides were evaluated for sensitivity and specificity. Clear agglutination was observed with UV-inactivated *B. anthracis* spores up to 10^5 spores per ml for all peptide-based LAT reagents (Fig. 3), establishing the detection limit at 10^5 spores per ml.

Specificity testing against UV-inactivated spores of *B. anthracis*, related *Bacillus* spp. (*B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. licheniformis*), and other Firmicutes (*Listeria monocytogenes* and *Staphylococcus aureus*) demonstrated

Table 3 Spore recovery in spiked soil and meat meal samples using the GABRI technique with different carrier media

Sample	Carrier media used	Spore count (concentrated in 100 μl)		Recovery %	Agglutination with LAT
		Count	Log unit		
Spiked soil sample	Sucrose solution	7×10^5	5.85	≈50%	Positive
	Deionized water with Triton X-100	3×10^4	4.48	≈40%	Mild positive
	PBS with Tween 20	9×10^6	6.95	≈60%	Strong positive
	Sucrose with Triton X-100	11×10^3	4.04	≈30%	Negative
	Sucrose with Tween 20	1×10^7	7.00	≈60%	Strong positive
Spiked meat meal sample	Sucrose solution	4×10^5	5.60	≈50%	Mild positive
	Deionized water with Triton X-100	9×10^3	3.95	≈30%	Mild positive
	PBS with Tween 20	2×10^7	7.30	≈70%	Strong positive
	Sucrose with Triton X-100	3×10^3	3.48	≈30%	Negative
	Sucrose with Tween 20	8×10^6	6.90	≈60%	Strong positive

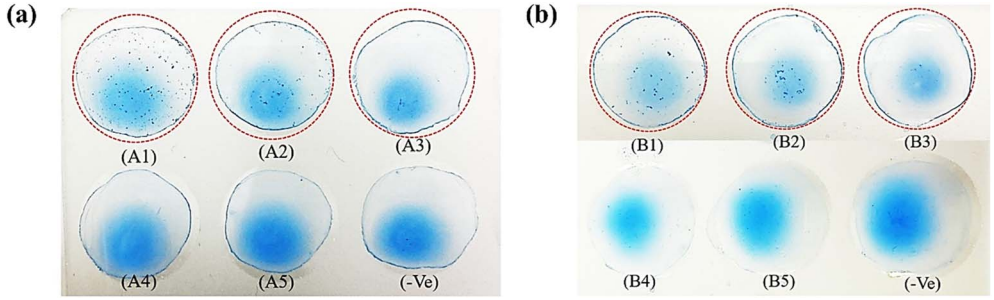

Fig. 6 Sensitivity of LAT with the spores recovered from spiked soil and meat meal. Images (a) spiked soil sample and (b) spiked meat meal sample. A1, B1: 10^{10} spores spiked samples. A2, B2: 10^{8} spore-spiked samples. A3, B3: 10^{6} spores spiked samples, A4, B4: 10^{4} spores spiked samples and A5, B5: 10^{2} spores spiked samples. –ve: negative control.

distinct agglutination exclusively with *B. anthracis* spores. Mild cross-reactivity was observed for EA-2 with *B. cereus* and *B. mycoides*, and for PA-2 with *B. cereus*, whereas PA-1 and EA-1 exhibited strong agglutination and high specificity (Fig. 4).

Evaluation with mixed spore suspensions containing different proportions of *B. anthracis* and other *Bacillus* spp. (100 : 0 to 0 : 100) Further confirmed the robustness of the assay. PA-1 and EA-1 LAT reagents produced visible agglutination in all mixtures containing *B. anthracis* spores, while no reaction occurred in suspensions containing only non-*anthracis Bacillus* spores. In contrast, PA-2 and EA-2 LAT reagents showed occasional mild non-specific reactions (Fig. 5).

The detection limit observed in the present study is comparable with previously reported LAT systems for anthrax detection. For example, PA peptide-based LAT assays detected formalin-treated vegetative cells at approximately 5×10^{4} cells per ml,[25] while monoclonal antibody-based LAT assays reported detection limits of 10^{5}–10^{6} spores per ml.[59] LAT systems targeting γDPGA have also demonstrated detection thresholds of 10–25 antigen units.[60] More recently, synthetic peptide-oriented IgY-based LAT assays showed specificity toward *B. anthracis* spores with a detection limit of around 10^{4} spores per ml.[6]

These findings indicate that peptide-directed IgY-based LAT reagents can provide a sensitive and specific platform for rapid detection of *B. anthracis* spores. Further improvements in assay sensitivity may be achievable by employing multiple antigenic peptides (MAPs) instead of single linear peptides, which have been shown to enhance immunogenicity and diagnostic performance.[61]

3.4 Determination of peptide specificity by indirect ELISA

An indirect ELISA, which is more sensitive than the LAT assay, was employed to further assess the specificity and potential cross-reactivity of IgY antibodies raised against the selected *B. anthracis*-specific peptides (PA-1, PA-2, EA-1, and EA-2). Based on OD_{492} cut-off values, *B. anthracis* spores showed positive reactions with all peptide-derived IgY antibodies. Mild cross-reactivity was observed for PA-2 and EA-2 antibodies with *B. cereus*, and for EA-2 with *B. mycoides*, whereas all other *Bacillus* species tested negative. Among the peptides evaluated, PA-1 and EA-1 demonstrated the highest specificity for *B. anthracis* spores, consistent with the specificity patterns observed in the LAT assay (SI Table S2 and Fig. S8). These observations are in agreement with earlier ELISA-based immunodiagnostic studies targeting anthrax-associated antigens. For instance, an ELISA based on poly-L-lysine capsular antigen reported 68% sensitivity and 100% specificity,[62] while monoclonal antibody-based sandwich ELISAs targeting PA and LF toxins achieved detection limits as low as 1 ng ml^{-1}.[63] Similarly, an indirect ELISA targeting PA83 and its cleaved form, PA63, demonstrated 100% specificity with a detection limit of 20 μg ml^{-1}.[64]

Collectively, these results confirm that peptide-derived IgY antibodies, particularly those targeting PA-1 and EA-1 epitopes, exhibit high specificity toward *B. anthracis* spores and represent

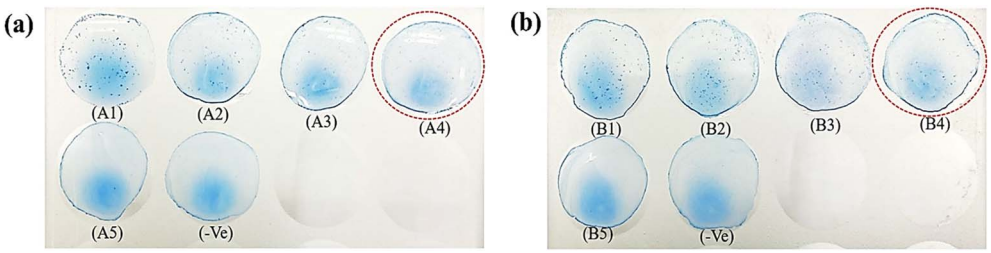

Fig. 7 Specificity of LAT with the spores recovered from spiked soil and meat meal with different proportions of spores. Images (a) represent a spiked soil sample, while (b) denotes a spiked meat meal sample. A1, B1: BA-100% spiked samples; A2, B2: BA-75% + OB-25% spore spiked samples. A3, B3: BA-50% + OB-50% spore spiked samples. A4, B4: BA-25% + OB-75% spiked samples. A5, B5: OB-100% spore spiked samples. –ve- negative control. (BA- *B. anthracis*. OB- other *Bacillus* spp.).

Table 4 Agglutination pattern of spores recovered from spiked soil and meat meal samples with different proportions of spores[a]

Sample	Spiked spore (10^{10} ml^{-1})	Spore recovery	Agglutination with LAT
Spiked soil (A)	BA-100% (A1)	1×10^8	Positive
	BA-75% + OB-25% (A2)	6×10^7	Positive
	BA-50% + OB-50% (A3)	3×10^7	Positive
	BA-25% + OB-75% (A4)	8×10^8	Mild positive
	OB-100% (A5)	5×10^8	Negative
Spiked meat meal (B)	BA-100% (B1)	11×10^7	Positive
	BA-75% + OB-25% (B2)	8×10^7	Positive
	BA-50% + OB-50% (B3)	6×10^8	Positive
	BA-25% + OB-75% (B4)	4×10^7	Mild positive
	OB-100% (B5)	8×10^8	Negative

[a] BA: *B. anthracis*, OB: other *Bacillus* spp. (*B. cereus*, *B. thuringiensis*,*B. mycoides*, *B. licheniformis*).

a reliable approach for immunodiagnostic detection; therefore, the PA-1 and EA-1 peptide-derived IgY antibody-based LAT assay was selected for further evaluation in spiking studies and field sample comparisons.

3.5 Evaluation of aqueous media for spore recovery and performance of LAT in spiked soil and meat meal samples

Spore recovery from soil and meat meal matrices was evaluated using the GABRI method with different aqueous carrier media. Among the tested media, PBS-Tween 20 and sucrose-Tween 20 yielded the highest recovery rates (~60–70%), followed by plain sucrose (~50%), whereas Triton X-100-based media resulted in comparatively lower recovery (~30–40%) (Table 3).

Spores recovered using PBS-Tween 20 and sucrose-Tween 20 produced strong agglutination with the optimized LAT assay, while only mild agglutination was observed with spores recovered using plain sucrose and none with Triton X-100-based media (SI Fig. S9). Based on both recovery efficiency and LAT performance, sucrose-Tween 20 was selected as the carrier medium for further experiments. These findings are consistent with previous reports indicating improved spore recovery when sucrose is combined with detergents, which help disrupt hydrophilic interactions between spores and soil particles.[40] Similar recovery efficiencies (≈60–70%) have been reported using related extraction approaches and peptide-based IgY LAT systems employing the GABRI technique.[6] Variations in recovery rates across studies may be attributed to differences in soil composition, pH, and organic matter content.[65]

The sensitivity and specificity of the developed LAT assay were further evaluated in sterile soil and meat meal samples spiked with varying concentrations of *B. anthracis* spores. Using the optimized GABRI method with sucrose-Tween 20 as the carrier medium, spore recovery ranged between 50–75% across both matrices. The LAT assay was able to detect up to 10^6 spores per gram of soil or meat meal sample (Fig. 6), establishing 10^6 spores per g as the detection limit under these matrix conditions. The detection limit observed in the spiking studies was 10-fold higher than that for plain spores, likely due to environmental interference from soil or meat meal matrices and variations in matrix pH.[66] Similar reductions in sensitivity have been reported in immunomagnetic–electrochemiluminescent detection of *B. anthracis* spores in soil matrices.[67]

Specificity was assessed using mixtures of *B. anthracis* and related *Bacillus* species spores in varying ratios (100 : 0 to 0 : 100). Clear agglutination was observed only in samples containing *B. anthracis* spores, regardless of the mixture ratio, confirming the specificity of the assay in complex matrices (Fig. 7 and Table 4).

The recovery efficiency and detection sensitivity observed in this study are comparable with previously reported approaches for detecting *B. anthracis* spores in environmental or food matrices. For example, Shields *et al.* in 2012,[43] reported recovery rates of 60–88% from food matrices using a stainless-steel mesh filtration system, while Thomas *et al.* in 2013,[68] achieved 80–87% recovery using immunomagnetic separation. Higher analytical sensitivity has been reported with advanced biosensor platforms such as surface plasmon resonance, which

Table 5 Screening of field samples

State	Type of sample	Total samples	Positives	
			PCR	LAT (IgY)
Tamil Nadu	Soil samples	59	2	2
	Feed supplement	21	0	0
	Total	80	2	2
Telangana	Soil samples	10	0	0
	Feed supplement	21	0	0
	Total	31	0	0
Maharashtra	Soil samples	19	0	0
	Feed supplement	2	0	0
	Total	21	0	0
Odisha	Soil samples	6	0	0
	Feed supplement	3	0	0
	Total	9	0	0
Karnataka	Soil samples	12	0	0
	Feed supplement	2	0	0
	Total	14	0	0
Andhra Pradesh	Soil samples	100	9	6
	Feed supplement	2	0	0
	Total	102	9	6
Grand total		257	11	8

Table 6 Comparative diagnostic efficacy of in-house developed LAT assays with WOAH-recommended PCR assay[a]

LAT developed with IgY antibody against peptide *vs.* PCR targeting *pag* (reference test)		PCR		
		Positive	Negative	Total
LAT developed with IgY antibody against the peptide	Positive	8 (a)	0 (b)	8 ($a + b$)
	Negative	3 (c)	246 (d)	249 ($c + d$)
	Total	11 ($a + c$)	246 ($b + d$)	257

[a] a: True positives; b: false positives; c: false negatives; d: true negatives; relative sensitivity $[(a/a + c) \times 100] = 72.7\%$; ; relative specificity $[(d/b + d) \times 100] = 100\%$; positive likelihood ratio = 0.999999; negative likelihood ratio = 0.27; positive predictive value $[(a/a + b) \times 100] = 100\%$; negative predictive value $[(d/c + d) \times 100] = 98.7\%$; Kappa value = 0.83 (perfect agreement); agreement (%) = 98.83%.

can detect single spores,[69] although such methods require specialized instrumentation. Differences in recovery efficiency across studies likely reflect variations in extraction methods, sample matrices, and processing conditions. Combining complementary recovery approaches has been suggested to further enhance spore detection efficiency, particularly in samples with low spore density.[65,70]

Overall, the optimized GABRI extraction combined with peptide-specific IgY-based LAT demonstrated reliable recovery and detection of *B. anthracis* spores in soil and feed matrices, supporting its potential application as a rapid and practical diagnostic approach for environmental surveillance.

3.6 Evaluation of the developed LAT with field samples

Among the 257 samples tested, 11 were positive by PCR, while 8 samples were positive by the LAT assay (Table 5). Diagnostic performance analysis using 2 × 2 contingency tables indicated that, when compared with PCR, the LAT assay exhibited a sensitivity of 72.7% and a specificity of 100%, with near-perfect agreement ($\kappa = 0.83$) (Table 6). The cost per LAT reaction was estimated to be ₹ 36.6 (approximately USD 0.42; SI Table S3), highlighting the economic feasibility of the assay for routine screening.

Comparable diagnostic performance has been reported for peptide-based LAT systems used in pathogen detection, with sensitivities and specificities ranging from 35–97% depending on the antigen target and assay configuration.[34,35] In the present study, LAT reagents based on IgY antibodies targeting PA-1 and EA-1 peptides demonstrated high specificity toward *B. anthracis* spores, with no false–positive reactions among PCR-negative samples.

Overall, the assay's simplicity, rapid turnaround time, and low operational cost make it a promising tool for on-site screening of *B. anthracis* spores in environmental and feed samples, particularly in resource-limited settings and endemic regions where rapid preliminary detection is critical for outbreak surveillance and control.

4 Conclusion

This study demonstrates that synthetic peptide-based immunodiagnostics constitutes a reliable and analytically robust approach for the selective detection of *Bacillus anthracis*. Peptides PA-1 and EA-1, derived from protective antigen and S-layer proteins, elicited high-titre, highly specific IgY antibodies with negligible cross-reactivity toward related *Bacillus* spp. The optimized LAT (200 μg ml^{-1} IgY) produced consistent and reproducible agglutination without added benefit at higher concentrations, while indirect ELISA independently confirmed peptide specificity. The use of peptide-derived IgY antibodies offers clear analytical advantages over conventional whole-cell antigens, including improved biosafety, reduced non-specific interactions, and enhanced assay reproducibility. The developed platform combines rapid response, operational simplicity, and low cost, supporting its applicability as a practical screening tool in environmental and field settings. Importantly, the multi-epitope peptide strategy enhances diagnostic reliability by reducing the likelihood of false negatives due to antigenic variability. Future work should focus on large-scale validation across diverse environmental and clinical matrices, as well as integration with portable or sensor-based analytical platforms to enable real-time, on-site detection. Overall, this work advances peptide-guided immunoassay design and provides a scalable, sensitive, and application-oriented framework for anthrax surveillance within a one health context.

Conflicts of interest

There are no conflicts to declare.

Data availability

All the data supporting this article have been included in the main text. The authors can provide data upon request.

Supplementary information (SI): SI Fig. S1: sporulation percentage of different *Bacillus* spp. at different time-points. SI Fig. S2a: *B. anthracis* spore preparation (Schaffer and Fulton Spore Staining). SI Fig. S2b: spores of different *Bacillus* spp. after 5–6 days of incubation (100X magnified). SI Fig. S3a: PCR targeting the *pag* gene with DNA extracted from inactivated and live spores. SI Fig. S3b: agglutination pattern of inactivated *B. anthracis* spores stored at different temperatures (After 2 weeks of storage). Fig. S4: HPLC report of PA-1 peptide. SI Fig. S5: mass spectrophotometry report of PA-1 peptide. SI Fig. S6: HPLC report of EA-1 peptide. Fig. S7: mass spectrophotometry report of EA-1 peptide. SI Fig. S8: specificity of the peptide-derived IgY antibodies using in-house-developed indirect ELISA. SI Fig. S9:

performance of LAT with the spores recovered through the GABRI technique using different carrier media. SI Table S1: details of the primers used in this study. SI Table S2: absorbance (OD) and cut-off values of in-house designed indirect ELISA. SI Table S3: cost of production of the LAT assay. See DOI: https://doi.org/10.1039/d6ra02624a.

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