Analytical Methods

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2024, **16**, 7242

Isothermal recombinase polymerase amplification and silver nanoparticle assay: a sustainable approach for ultrasensitive detection of *Klebsiella pneumoniae*†

Naresh Patnaik,‡ Nidhi Orekonday‡ and Ruchi Jain Dey *

Our study addresses the urgent need for effective detection of *Klebsiella pneumoniae*, a recognized threat by the World Health Organization (WHO). Current challenges in managing *K. pneumoniae* infections include the lack of rapid and affordable detection tools, particularly in resource-limited point-of-care (POC) settings. To tackle this, we developed an innovative molecular detection pipeline combining three POC-compatible methods. Firstly, we employed Insta DNA™ card-based sample collection and DNA extraction for simplicity and ease of use. Next, we utilized recombinase polymerase amplification (RPA) targeting the *Klebsiella* hemolysin gene, *khe*, specific to the *K. pneumoniae* species complex (KpSC). Finally, we integrated a silver nanoparticle (AgNP) aggregation assay for visual detection, offering a rapid, sensitive, and specific method capable of detecting as few as ~3 bacteria of *K. pneumoniae* within ~45 minutes. This approach eliminates the need for complex equipment, making it highly suitable for field and resource-limited POC applications. Moreover, our method introduces an environmentally significant detection strategy. The method developed minimizes chemical reagent usage and reduces the carbon footprint associated with sample transportation. Furthermore, our method reduces waste compared to the traditional detection techniques, offering a safer alternative to ethidium bromide or other DNA dyes which are often genotoxic and mutagenic in nature. Silver nanoparticles, being environmentally safer, can also be recycled from the waste, contributing to sustainability in nanoparticle production and disposal. Overall, our technique presents a promising solution for detecting *K. pneumoniae* in various settings, including environmental, water, and food samples, as well as industrial or hospital effluents. By aligning with global efforts to improve public health and environmental sustainability, our approach holds significant potential for enhancing disease management and reducing environmental impact.

Received 27th May 2024
Accepted 19th September 2024

DOI: 10.1039/d4ay00993b

rsc.li/methods

Introduction

The escalation of antimicrobial resistance (AMR) constitutes a significant global health threat, particularly within the notorious ESKAPE group of pathogens. This acronym encompasses six-opportunistic bacterial pathogens with high capabilities of acquiring antibiotic resistance: *Enterococcus species* (spp.), *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.[1] Within the ESKAPE group of pathogens, the World Health Organization (WHO) recognizes *K. pneumoniae* as a significant pathogen of concern, primarily due to its association with increasing AMR.[1–3] *K. pneumoniae* species complex (KpSC) comprises seven phylogroups termed as phylogroups Kp1 to Kp7 (ref. 4 and 5) (Table S1†). KpSC is a major contributor to nosocomial infections, especially in vulnerable or immune-compromised individuals leading to diverse pathologies such as necrotizing pneumonia, urinary tract infections (UTIs), wound or surgical site infections, bloodstream infections, septicaemia, meningitis, and pyogenic liver abscesses.[6–9] *Klebsiella* infections are also important in the context of maternal and child health causing complications such as chorioamnionitis, increased likelihood of pre-term labour/birth,[10] neonatal infections leading to increased infant mortality.[11,12] Hence, preventive measures and early detection of *Klebsiella* is paramount for timely intervention and effective management of these infections in maternal and child healthcare settings.

Early intervention facilitates prompt initiation of appropriate medical measures, reducing the risk of severe complications and preventing transmission. Conventional methods for detecting *K. pneumoniae*, such as culture-based and biochemical techniques, are time-consuming and provide

Department of Biological Sciences, BITS Pilani Hyderabad Campus, Telangana State 500078, India. E-mail: ruchij80@hyderabad.bits-pilani.ac.in

† Electronic supplementary information (ESI) available. See DOI: https://doi.org/10.1039/d4ay00993b

‡ Equal contribution as co-first authors.

Table 1 List of visual detection methods reported previously for the molecular detection of *K. pneumoniae*

Sl. no.	Method of amplification	Method of detection	Sensitivity	Time complexity of detection	References
1	PCR	AGE	1.2×10^2 bacteria	~4–5 hours	16
2	Real-time PCR	Fluorescence	15 bacteria	~2 hours	4
3	Real-time PCR	Fluorescence resonance energy transfer (FRET)	10 bacteria	~2 hours	17
4	PCR	Gold nanoparticles	16×10^1 bacteria (1 pg gDNA)	~2 hours	18
5	LAMP	AGE	1 bacterium	~25 min (LAMP) + 1 hour AGE	19
6	LAMP	CRISPR-based detection	16×10^1 bacteria (1 pg gDNA)	~1 hour	20
7	MCDA	Gold nanoparticle-LFS	16 bacteria (100 fg gDNA)	~30 min (estimated, not reported)	21
8	RPA	LFS	10^3 bacteria	~15 min	22
9	RPA	LFS	16×10^3 bacteria (0.1 ng μl^{-1})	~33 min	23
10	Real-time RPA	Fluorescence probes	100–1000 bacteria	~12 min	24
11	RPA	AGE	10^2–10^3 bacteria	~1 hour	25

Table 2 Oligonucleotide primers used for molecular detection of *K. pneumoniae*[a]

Si no.	Target gene (amplicon size)	Primer sequence (5′ → 3′)	PCR conditions[b]
1	*khe* gene (404 bp)	(F) CGGGATTGAGCGGGTAATAA (R) GATGAAACGACCTGATTGCATTC	95 °C, 30 s; 64 °C, 30 s; 72 °C, 30 s
2	*uge* gene (222 bp)	(F) CATATTCTTCTGCGCTTCCATTC (R) AAGCGCGACTTCACCTATATC	95 °C, 30 s; 64 °C, 30 s; 72 °C, 30 s

[a] All the primers were designed using PrimerQuest tool (Integrated DNA Technologies) in this study. [b] Annealing temperatures obtained using gradient PCR.

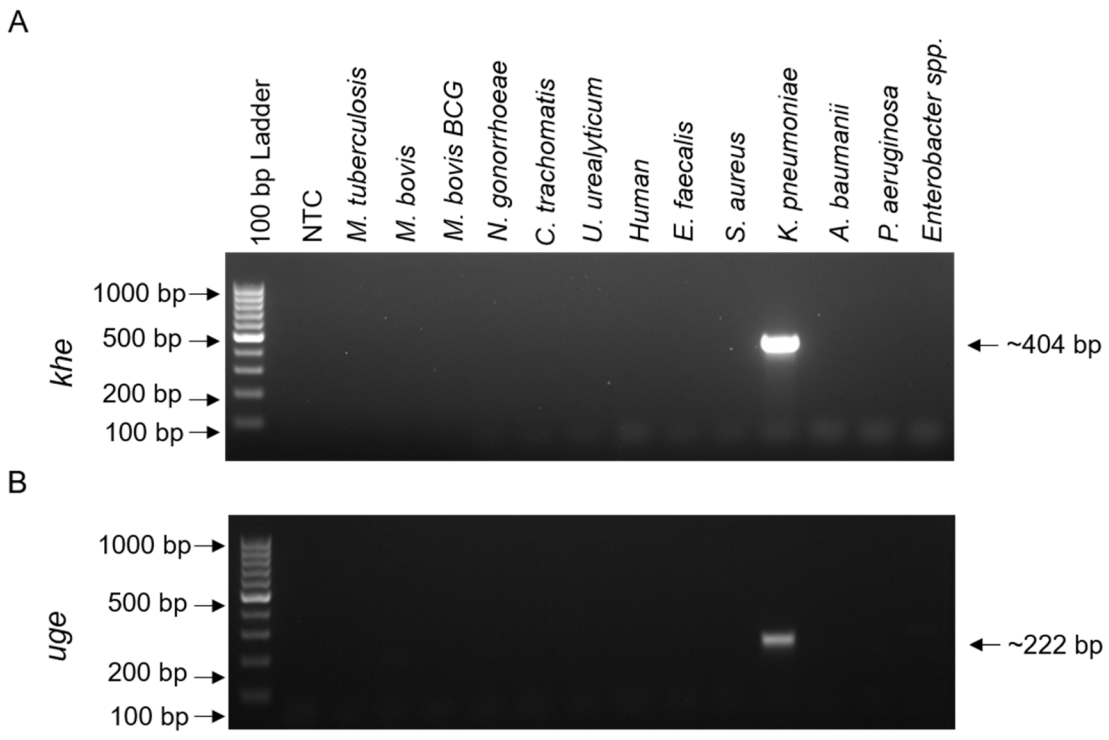

Fig. 1 Specificity and cross-reactivity verification of primers designed for the molecular detection of *K. pneumoniae*. The agarose gel images depict specificity of designed primer sets, specific to two genomic regions of *K. pneumoniae*, namely (A) *khe* (~404 bp) and (B) *uge* genes (~222 bp), using PCR following a kit based gDNA isolation. The controls include-no template control (NTC), human DNA, and gDNA obtained from 11 major pathogens associated with the human host. The gel images are a representation of three experimental replicates.

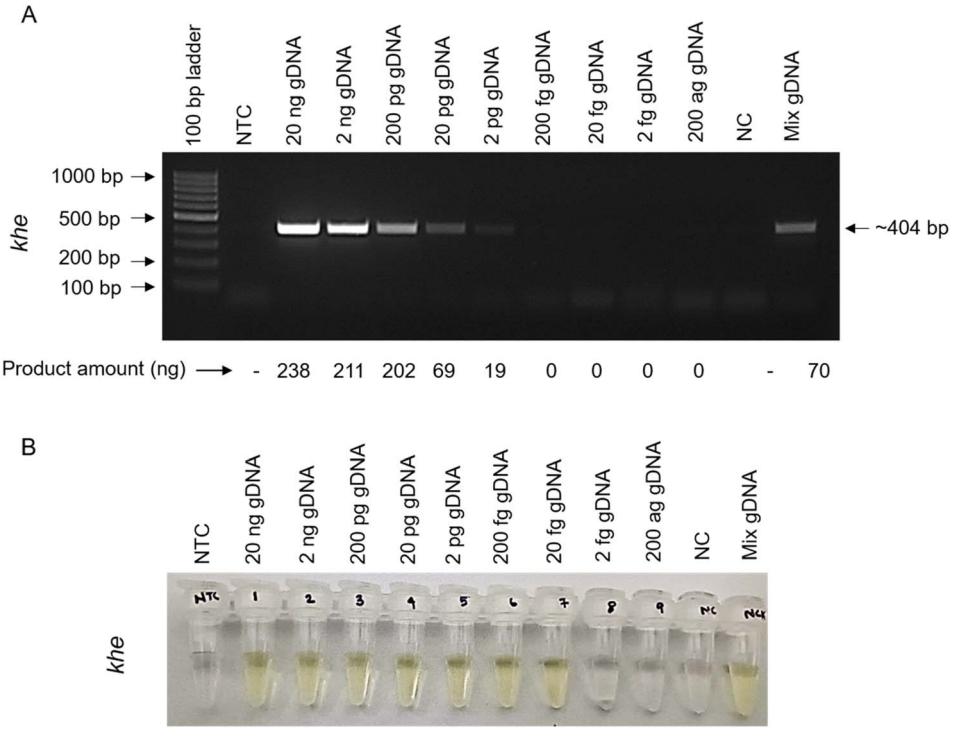

Fig. 2 Sensitivity of molecular detection of *K. pneumoniae* using PCR-AGE. The figure depicts a sensitivity analysis of PCR-AGE-based molecular detection of *K. pneumoniae* using primers specific to genomic region (A) *khe* gene and (B) AgNP detection of the PCR amplicons. The labels on top of the gel indicate the amount of the genomic DNA (isolated using gDNA isolation kit) used per PCR reaction. The controls include-no template control (NTC) and negative control (NC), a blend of gDNAs obtained from human and 11 different pathogens associated with the human host. Mix gDNA contains a mix of NC and genomic DNA of *K. pneumoniae*. The PCR-AGE assay for *Khe* gene shows a sensitivity of detection of $\sim 33 \times 10^1$ bacteria of *K. pneumoniae*. The numbers at the bottom of the gel image correspond to the relative quantification of the PCR amplicons in ng (w.r.t 100 bp ladder), done using ImageJ software as described in methods. The gel images are a representation of four experimental replicates. (B) The InstaDNA-PCR-AgNP assay displays a detection sensitivity of ~3 bacteria for the target genes.

limited information on abundance and strain diversity within samples, with uncertain sensitivity.[4,5,13] The recent introduction of whole metagenomics sequencing in clinical diagnostics has significantly enhanced diagnostic reliability[13,14] compared to traditional culture-based methods, offering comprehensive insights into antibiotic resistance profiles, virulence features, and evolutionary analysis of bacterial strains.[13,15] Nevertheless, the exorbitant cost, time involvement and requirement of highly skilled individuals to perform and analyse the results currently restricts its routine application in diverse healthcare settings. In contrast, PCR-based assays, particularly real-time quantitative PCR(RT-qPCR),[4] have shown 100% sensitivity and specificity,

Table 3 Advantages of InstaDNA card as POC compatible DNA isolation method

Parameters	Advantages of InstaDNA card
Sample collection	Sample collection in the card itself
Sample pre-processing	No pre-processing is necessary
Sample storage and transportation	InstaDNA cards allow room temperature storage and transportation
Equipment necessary for DNA isolation	Heat block for DNA isolation
Compatibility with PCR and RPA amplification techniques	Yes. Discs cut from the InstaDNA card containing sample can be directly used for PCR, without the need for DNA elution. DNA elution step is necessary before RPA
Compatibility with AgNP assay	Yes
Cost	Low cost. Sample collection, storage, transportation is done on the InstaDNA cards at room temperature. No requirement for sample pre-processing and equipment. Recurring cost involves only InstaDNA cards (<1$ per sample) and a simple heat block or water bath
Time for DNA extraction	~15–30 minutes. ~2 minutes for DNA elution. Samples collected on the InstaDNA cards are usually air dried, which takes ~15–30 minutes
DNA extraction & amplification efficiency	High

with a limit of detection of ~15 genomes (Table 1). However, the cost of detection is extremely high due to requirement of quantitative PCR (qPCR) equipment. Subsequent developments introduced isothermal amplification methods, including loop-mediated isothermal amplification (LAMP),[19,20] recombinase polymerase amplification (RPA)[22–25] and multiple cross displacement amplification (MCDA),[21] for rapid *K. pneumoniae* detection without the requirement of expensive thermal cyclers or qPCR. LAMP alone or in combination with clustered regularly interspaced short palindromic repeats (CRISPR) and MCDA combined with lateral flow strip (LFS) has demonstrated comparable sensitivity (1–20 bacteria) with respect to qPCR.

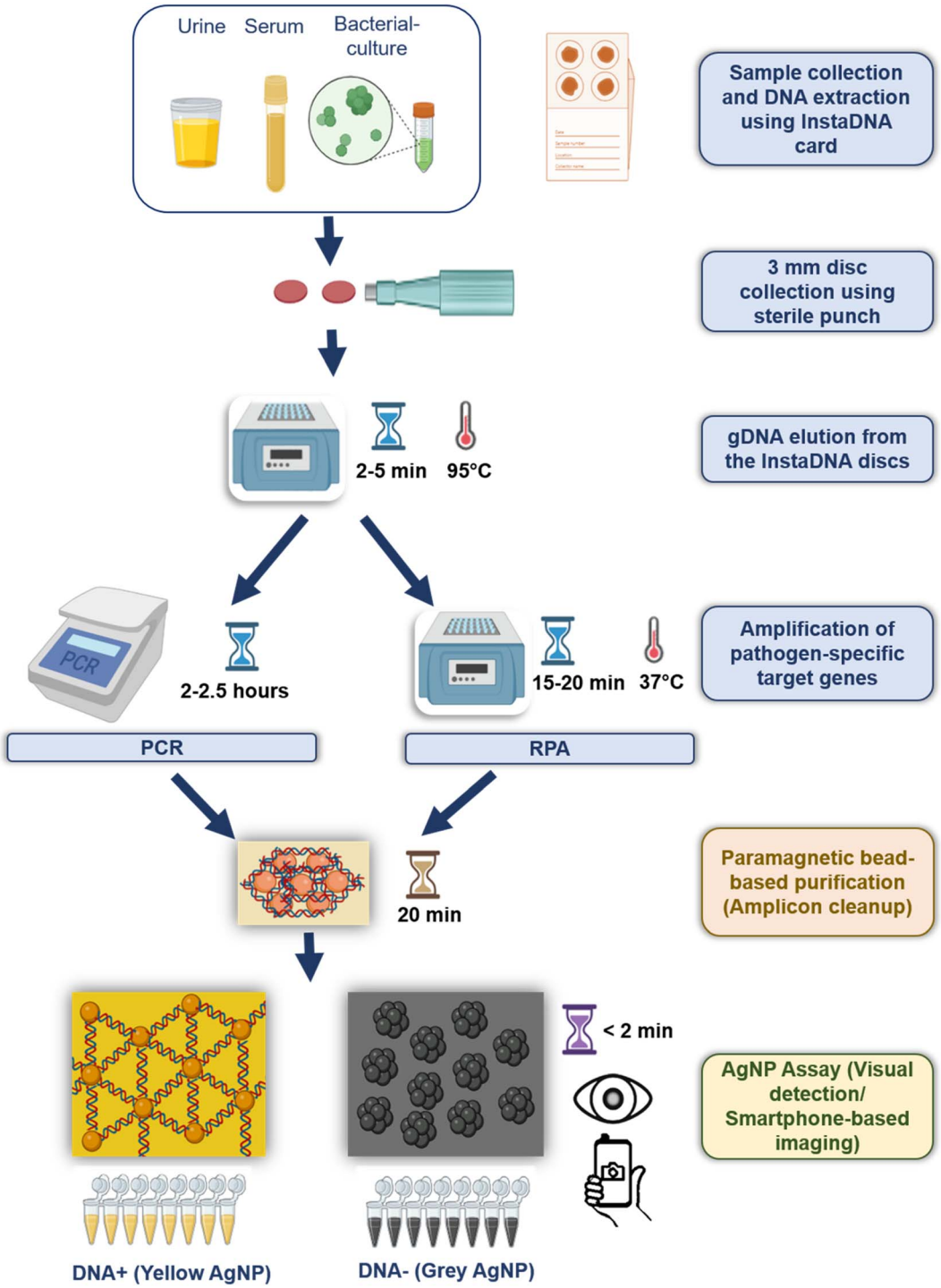

Fig. 3 Schematic diagram for molecular detection of pathogens. The figure depicts schematic representation of the molecular detection pipeline developed using combination of three POC-compatible methods; InstaDNA card-based sample collection and DNA extraction, RPA-based isothermal amplification of the target gene, and AgNP assay for visual- or camera-based detection.

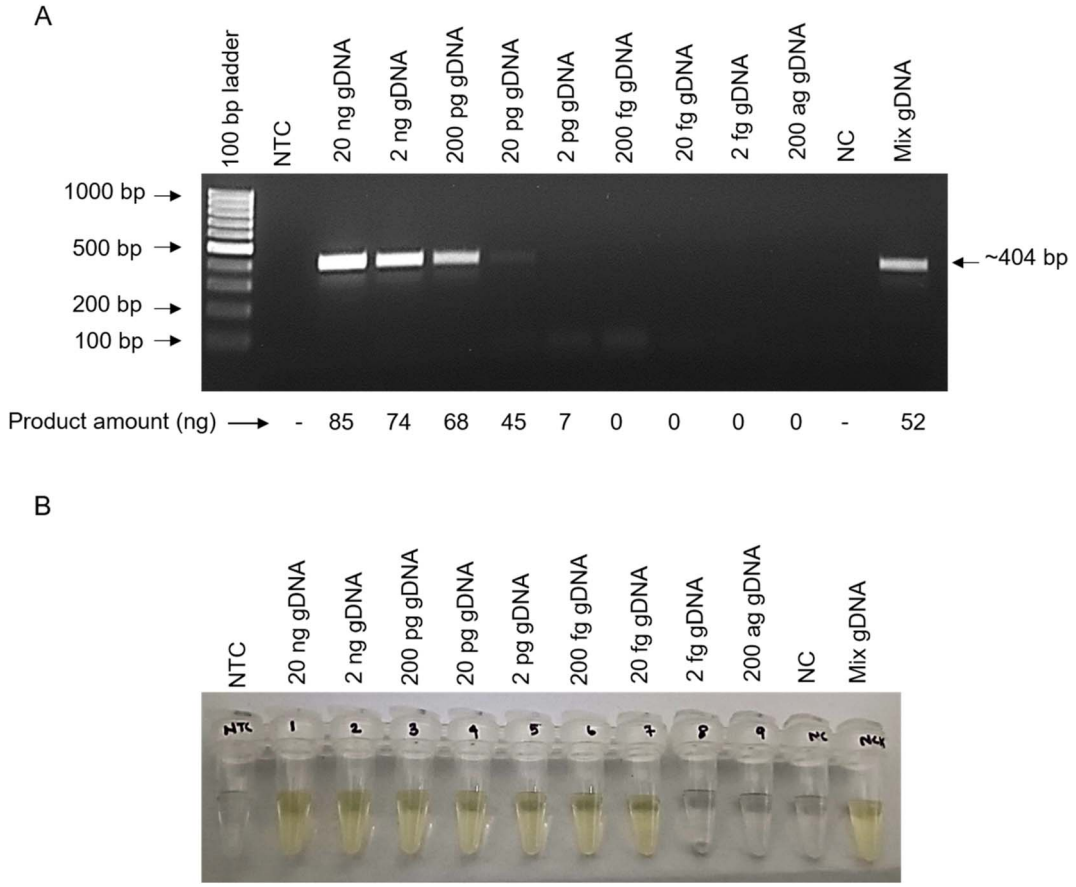

Fig. 4 Sensitivity of molecular detection of *K. pneumoniae* by InstaDNA-PCR-AgNP assay. AgNP assay is used for visual molecular detection of InstaDNA-PCR products obtained from PCR performed using *khe* gene primers. (A) The InstaDNA-PCR-AGE assay shows a sensitivity of detection of $\sim 33 \times 10^2$ bacteria of *K. pneumoniae*. The numbers at the bottom of the gel image correspond to relative quantification of the PCR amplicons in ng (w.r.t to 100 bp ladder), done using ImageJ software as described in methods. (B) The InstaDNA-PCR-AgNP assay displays a detection sensitivity of ~3 bacteria for the target gene. The images are the representation of four experimental replicates. The controls include-no template control (NTC) and negative control (NC), a blend of gDNAs obtained from human and 11 different pathogens associated with the human host. Mix gDNA contains a mix of NC and genomic DNA of *K. pneumoniae*.

These methods achieve detection within 60–90 minutes under isothermal conditions (~60–65 °C)[19–21] (Table 1). In comparison, RPA provides further advantage of rapid (15–30 min) isothermal amplification at lower temperatures (~37–42 °C) permitting quicker detection.[26] RPA when combined with LFS is capable of detecting as low as 100–1000 bacteria, highlighting an improved sensitivity of detection post RPA-based DNA amplification.[22,23] RPA has been applied in recent past for developing non-instrumented point-of-care (POC) detection for several human,[27] animal[28] and plant pathogens.[29] For instance, RPA-based detection was developed for HIV-1 and plant viruses harnessing the body heat.[27,29]

Despite the advancements in a wide variety of methods of isothermal amplification under ambient settings, visualization of the amplified products still remains a major constraint in POC setting. A recent study highlights utility of tailor-made plasmonic aptamer-gold nanoparticle (AuNP) assay for detection of *Klebsiella* without the need of amplification, however, the sensitivity of this methods is ~3400 bacteria.[18] However, the high cost associated with fluorescent primer probes, aptamers, additional reagents like CRISPR, LFS and AuNP presents accessibility challenges for low-income and resource-poor settings, highlighting the need for cost-effective visual detection tools. Another hurdle in POC settings is safe and simple methods of sample collection, storage, transport and genomic DNA (gDNA) extraction for molecular detection. InstaDNA card have recently been developed to overcome these challenges. This card consists of a special filter paper impregnated with a proprietary formula containing reagents that promote cell lysis and protein denaturation with subsequent release of nucleic acids that are entrapped within the matrix of the card and stabilized at room temperature, allowing long-term storage and ensuring DNA integrity for downstream molecular detection.[30–33] Many advantages have been described for InstaDNA card, including low-cost, simple extraction protocols, easy transportation, minimal storage space and no special infrastructure being required.[33] However, there are no previous reports for application of InstaDNA cards for *K. pneumoniae* detection.

This study offers POC friendly molecular diagnostic pipeline combining three methods, (a) InstaDNA card, for easy sample collection and DNA extraction (b) RPA-based isothermal amplification in addition to PCR (c) application of silver nanoparticle

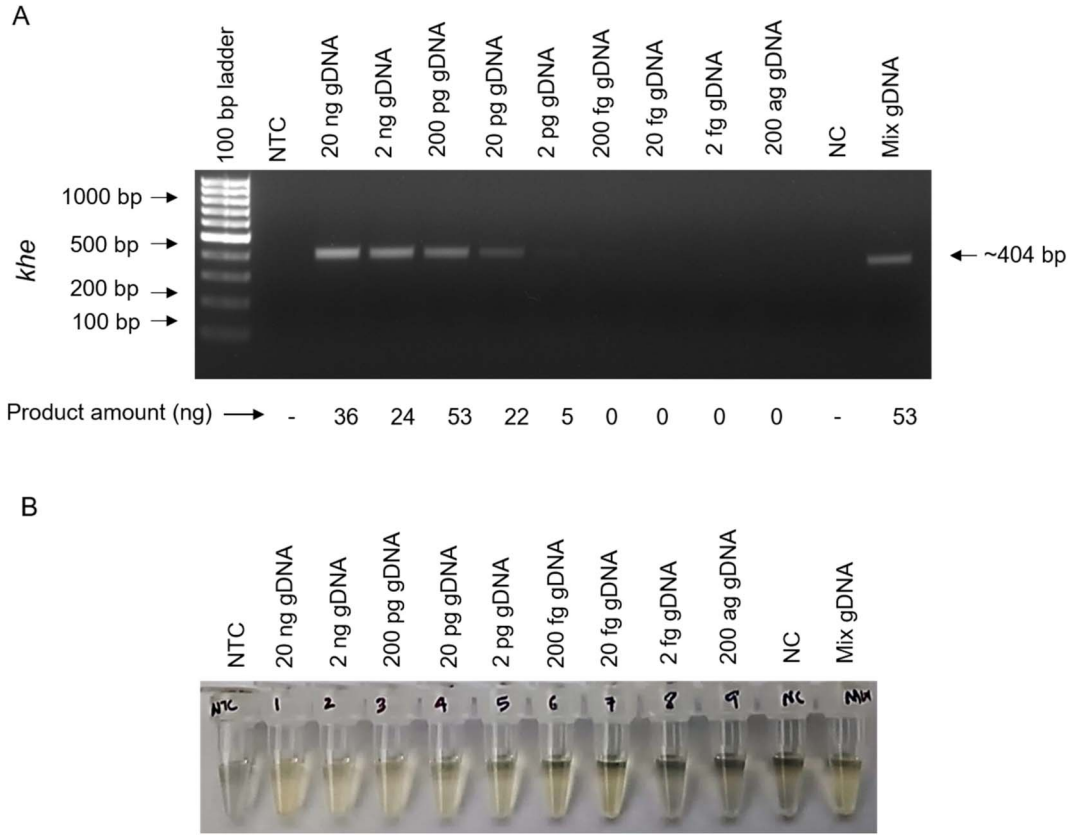

Fig. 5 Sensitivity of molecular detection of *K. pneumoniae* by RPA-AGE and RPA-AgNP assay using unique target sequence in *khe* gene. The figure depicts, (A) AGE-based and (B) AgNP assay-based detection of DNA amplified using RPA employing primer specific to *khe* gene (~404 bp) of *K. pneumoniae*, followed by magnetic bead-based purification. RPA-AGE shows a sensitivity of ~33 × 10^1 bacteria and RPA-AgNP assays shows detection sensitivity of ~3 bacteria of *K. pneumoniae*. Controls include-no template control (NTC) which consists of sterile water instead of template and negative control mix (NC), a mix of gDNAs obtained from human and 11 different pathogens associated with the human host. Mix gDNA contains a mix of NC and genomic DNA of *K. pneumoniae*. The images are a representation of three experimental replicates. The numbers at the bottom of gel image, correspond to the relative quantification of the RPA amplicons in ng (w.r.t to 100 bp ladder), done using ImageJ software as described in methods.

(AgNP) aggregation assay developed in our laboratory[34] for rapid, ultrasensitive visual molecular detection of *K. pneumoniae*. This unique combination is particularly suitable for *K. pneumoniae* detection in resource-poor settings without the need of costly thermal cycler and equipment like centrifuge or qPCRs and is compatible with clinical samples like serum and urine. The integration of these methods is anticipated to address the clinical needs to mitigate the impact of *Klebsiella* infections.

Results

Specific molecular detection of *K. pneumoniae*

In this study, we designed primers specific to two virulence gene, namely, *Klebsiella* hemolysin gene (*khe*)[35,36] and uridine diphospho (UDP)-D-galacturonate 4-epimerase gene (*uge*)[37] (Table 2). These two loci are unique to *K. pneumoniae* (Fig. 1A and B). We then elucidated the sensitivity of molecular detection of the bacteria using PCR followed by agarose gel electrophoresis (AGE) (called PCR-AGE hereafter) with all the primer sets. PCR-AGE performed using the highly specific primer sets against *khe* and *uge* genes, showed detection sensitivity of 33 × 10^1 (Fig. 2A) and 33 × 10^3 (ESI Fig. S1†) *K. pneumoniae* bacteria, respectively. Further, using *in silico* BLAST analysis we show that the primers sets designed against *khe* and *uge* can detect all the phylogroups of *KpSC* (Kp1 to Kp7) (ESI Table S1†). Given the low sensitivity of *uge* based PCR compared to *khe* (Fig. 2A and ESI Fig. S1†), hereafter we exclusively utilized *khe* gene target for comparative assessment of various POC molecular detection strategies for *K. pneumoniae*.

Since AGE requires equipment for UV-based visualisation, we next assessed the utility of an equipment-free and naked eye visual detection method, namely, AgNP assay following PCR. In addition, we also compared the sensitivity of detection of this method with respect to PCR-AGE. PCR-AgNP assay, show highly sensitive and specific detection of as low as ~3 bacteria (Fig. 2B).

InstaDNA card-based sample collection and DNA extraction followed by PCR based amplification and visual detection of *K. pneumoniae* by AgNP assay

Due to several advantages of InstaDNA card pertaining to their ease of sample collection, storage, logistics, cost and time

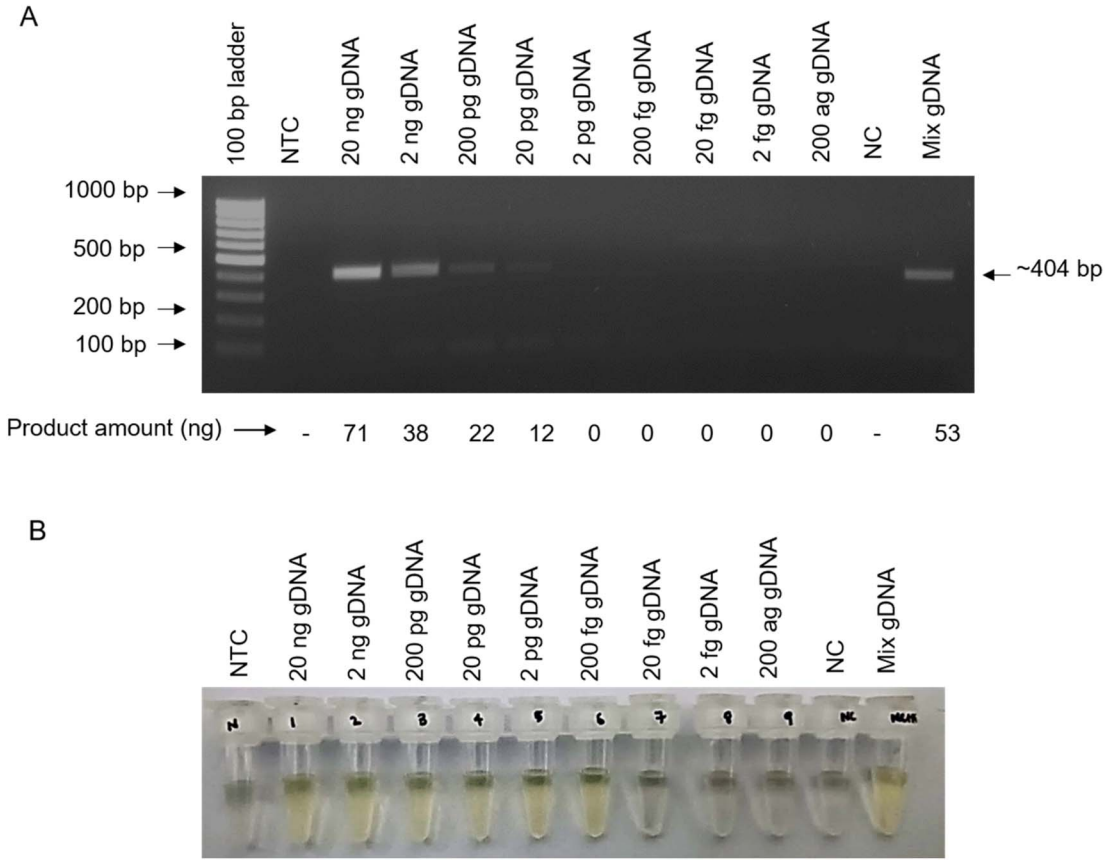

Fig. 6 Sensitivity of molecular detection of *K. pneumoniae* in serum sample spiked with bacterial gDNA by InstaDNA-PCR-AgNP assay. AgNP assay is used for visual molecular detection of InstaDNA-PCR products obtained from PCR performed using *khe* gene primers. (A) The InstaDNA-PCR-AGE assay shows a sensitivity of detection of $\sim 33 \times 10^2$ bacteria of *K. pneumoniae*. (B) The InstaDNA-PCR-AgNP assay displays a detection sensitivity of ~33 bacteria. Controls include-no template control (NTC) which consists of eluent obtained from blank InstaDNA discs blotted with sterile water and negative control (NC), a mix of gDNAs obtained from human and 11 different pathogens. Mix gDNA contains a mix of NC and genomic DNA of *K. pneumoniae*. The numbers at the bottom of the gel image, correspond to the relative quantification of the PCR amplicons in ng (w.r.t to 100 bp ladder), done using ImageJ software as described in methods.

effectiveness, efficiency of extraction without need of any high-end equipment (Table 3), compatibility with various amplification techniques and AgNP visual detection assay, we found InstaDNA cards to be most suited for POC application.[30–33] Hence, we next assessed the utility of InstaDNA card-based DNA isolation for PCR based molecular detection of *K. pneumoniae* as shown in Fig. 3.

InstaDNA-PCR-AGE combination shows a sensitivity of detection as low as $\sim 33 \times 10^2$ bacterial cells of *K. pneumoniae* when *khe* gene is used as the gene targets for PCR amplification (Fig. 4A). The advantage of using PCR, following an InstaDNA card-based DNA extraction is that we do not require any additional elution step, as elution is automatically favoured during the initial denaturation step performed during the PCR cycles. AgNP assay following InstaDNA-PCR using primer targeting *khe* gene (InstaDNA-PCR-AgNP) assay (Fig. 4B) showed highly sensitive and specific detection as low as ~3 bacteria similar to that observed in case of PCR-AgNP (Fig. 2B). Altogether, we report highly sensitive and specific detection of *K. pneumoniae* utilizing AgNP following PCR and InstaDNA-PCR.

POC compatible ultrasensitive molecular detection pipeline for *K. pneumoniae* using RPA and AgNP assay

Since PCR requires a high-end thermocycler which is often unavailable in resource poor POC settings, we next assessed the utility of isothermal amplification method using RPA, that performs DNA amplification under ambient temperature (25–42 °C) and can be conducted either on table-top or in an inexpensive heat-block or water-bath (Fig. 3). We performed the RPA amplification of *khe* gene of *K. pneumoniae* at 37 °C (Table 2), following standard gDNA isolation. We then compared two methods for visualization of amplified DNA products obtained on RPA, namely, AGE and AgNP assay (called as RPA-AGE and RPA-AgNP assay, respectively, hereafter). The RPA-AGE utilizing *khe* gene shows a detection sensitivity of $\sim 33 \times 10^1$ bacteria (Fig. 5A).

We next assessed the sensitivity of DNA detection using RPA-AgNP assay. AgNP dramatically improves visual detection of DNA obtained on amplification of *khe* gene with sensitivity as low as ~3 bacteria when compared to AGE ($\sim 33 \times 10^1$ bacteria) (Fig. 5A and B). AgNP assay thus, is suitable for molecular

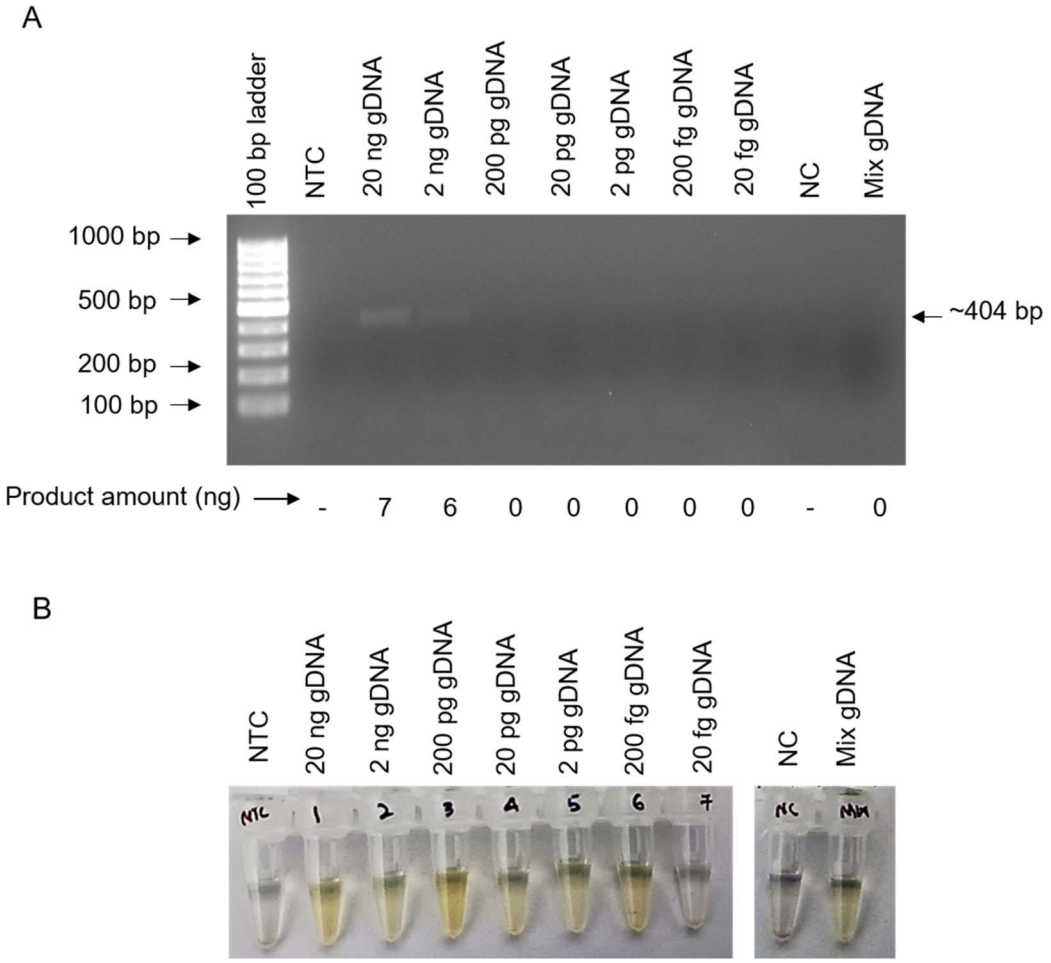

Fig. 7 Sensitivity of molecular detection of *K. pneumoniae* in serum sample spiked with bacterial gDNA by InstaDNA-RPA-AgNP assay. AgNP assay is used for visual molecular detection of InstaDNA-RPA products obtained from RPA performed using *khe* gene primers. (A) The InstaDNA-RPA-AGE assay shows a sensitivity of detection of $\sim 33 \times 10^4$ bacteria of *K. pneumoniae*. The numbers at the bottom of the gel image, correspond to the relative quantification of the RPA amplicons in ng (w.r.t to 100 bp ladder), done using ImageJ software as described in methods. (B) The InstaDNA-RPA-AgNP assay displays a detection sensitivity of ~33 bacteria for both the target genes. Controls include-no template control (NTC) which consists of eluent obtained from blank InstaDNA discs blotted with sterile water and negative control mix (NC), a mix of gDNAs obtained from human and 11 different pathogens. Mix gDNA contains a mix of NC and genomic DNA of *K. pneumoniae*.

Table 4 Limit of detection of *Klebsiella pneumoniae* in serum sample using InstaDNA-PCR-AGE, InstaDNA-PCR-AgNP, InstaDNA-RPA-AGE and InstaDNA-RPA-AgNP

Si. no	Input amount of DNA	Input amount of bacteria	InstaDNA-PCR-AGE	InstaDNA-PCR-AgNP	InstaDNA-RPA-AGE	InstaDNA-RPA-AgNP
1	20 ng	$\sim 33 \times 10^5$	+	+	+	+
2	2 ng	$\sim 33 \times 10^4$	+	+	+	+
3	200 pg	$\sim 33 \times 10^3$	+	+	−	+
4	20 pg	$\sim 33 \times 10^2$	+	+	−	+
5	2 pg	$\sim 33 \times 10^1$	−	+	−	+
6	200 fg	~33	−	+	−	+
7	20 fg	~3	−	−	−	−
8	2 fg	$\sim 3 \times 10^{-1}$	−	−	−	−
9	200 ag	$\sim 3 \times 10^{-2}$	−	−	−	−

detection post-RPA-based DNA amplification of the target genes and offers a useful alternative to AGE-based detection following RPA-based isothermal amplification techniques.

We then validated the compatibility of the InstaDNA-PCR-AGE & AgNP and InstaDNA-RPA-AGE & AgNP detection workflows in clinical settings. To simulate the protein-rich environment of clinical samples, we spiked serum from a healthy subject with varying amounts of *K. pneumoniae* gDNA on 3 mm InstaDNA card discs.

These discs were subsequently used for downstream PCR and RPA-based DNA amplification, followed by visualization using AGE (Fig. 6A and 7A) and AgNP (Fig. 6B and 7B), respectively. Additionally, all four assays were also validated using urine samples (ESI Fig. S2 and S3†). The results support the clinical applicability of InstaDNA-PCR-AgNP and InstaDNA-RPA-AgNP.

Discussion

Our study addresses the critical need for rapid, affordable, and effective detection of *Klebsiella pneumoniae*, a pathogen recognized as a significant threat by the WHO. Current diagnostic challenges include the lack of tools that are both cost-effective and suitable for POC settings, particularly in resource-limited environments. To overcome these challenges, we developed an innovative detection pipeline that integrates three POC-compatible methods: InstaDNA™ card-based sample collection and DNA extraction, RPA targeting the *khe* gene specific to KpSC, and an AgNP aggregation assay for visual detection.[34]

Our combined approach offers several key advantages. The InstaDNA™ card allows for simple and safe sample collection, storage, and transport, providing instant DNA extraction without the need for sophisticated equipment or additional reagents. The RPA method, targeting the *khe* gene, provides rapid amplification, while the AgNP assay facilitates visual detection, capable of identifying as few as 3 bacteria within approximately 45 minutes. This system eliminates the need for expensive thermal cycling equipment, centrifuges, and gel documentation facilities, making it highly suitable for field and POC applications. Furthermore, the non-labelled primer sets we developed are compatible with both PCR and RPA, offering flexibility and cost saving. RPA and InstaDNA card further offer multiple benefits enlisted in Tables 1 and 3.[4,16–25]

Importantly, our method aligns with environmental sustainability goals. The reduced use of hazardous dyes like ethidium bromide, along with the potential for recycling magnetic beads and silver nanoparticles, contributes to a lower environmental impact by eliminating the need for harmful chemicals. This approach not only supports global public health efforts but also promotes sustainable diagnostic practices.

AgNP assays present significant advancements in diagnostic capabilities, especially in resource-limited settings. Citrate-stabilized AgNPs are particularly advantageous due to their enhanced stability against photo-oxidation, which ensures a consistent and reliable signal over time. This stability, combined with their cost-effectiveness compared to gold nanoparticles, makes AgNPs a practical choice for DNA detection assays.[34] The unique properties of citrate-stabilized AgNPs, including their colorimetric response, offer a simple and visual means of detection without requiring complex instruments, making the method accessible even in POC settings. Furthermore, when coupled with isothermal amplification techniques like LAMP and RPA, the label-free AgNP aggregation approach could revolutionize POC diagnostics. These techniques could drastically reduce the cost, time, and complexities associated with diagnosis of infectious diseases, potentially improving the management of the disease in under-resourced areas. While visual detection is promising, it is important to note that it may not provide quantitative results, which are crucial for monitoring disease progression and treatment response. The integration of AgNP-based assays with isothermal amplification methods promises to enhance diagnostic capabilities in challenging settings such as hospitals, war zones, sewage treatment plants, food, water and pharmaceutical industries, remote or resource-poor areas, environmental monitoring sites, and even in-field testing during disease outbreaks or bioterrorism threats.

While our *in silico* analysis identified primers against *khe* and *uge* are suitable for detecting all seven phylogroups of *K. pneumoniae*, our experiments were limited to a laboratory strain (MTCC 109) and have not yet been validated on other phylogroups and in clinical settings. To address this, we conducted experiments using clinical mimic samples to simulate clinical conditions. These experiments demonstrate the effectiveness of the RPA and AgNP assays in serum and urine, further validating the method's potential for real-world clinical applications. Table 4 and ESI Table S3,† highlight the detection sensitivity of *K. pneumoniae* in clinical mimics. Future research in this direction will make this tool more robust and affordable for widespread applications.

Conclusion

This study presents an ultrasensitive, rapid, and cost-effective method for specific molecular detection of *K. pneumoniae* by combining Insta-DNA card-based DNA extraction, RPA isothermal amplification, and AgNP visual detection. We developed unique primers targeting the virulence gene *khe* that are compatible with both PCR and RPA assays. The method eliminates the need for expensive equipment, making it highly suitable for point-of-care (POC) settings with a sensitivity as low as ~3 bacteria per sample and a total cost under $5. Experiments using clinical mimic samples demonstrate the effectiveness of the PCR and RPA along with AgNP visual detection assays in serum and urine, further validating the method's potential for real-world clinical applications.

Materials and methods

Ethics statement

The study was conducted in accordance with the guidelines and approval of the Institutional Biosafety Committee (BITS/IBSC/2019-1/7 and BITS/IBSC/2019-1/8), and on ethical approval of

the Institutional Human Ethics Committee (IHEC), the Institutional Review Board (IRB) of Birla Institute of Technology and Science (BITS), Hyderabad (IHEC approval number: BITS-HYD/IHEC/2022/01) with appropriate biosafety level 2 measures.

Reagents and bacterial strains

All reagents used in this study were obtained from Himedia Laboratories Pvt. Ltd, unless specified otherwise. The oligonucleotide primers listed in Table 2 were purchased from Sigma-Aldrich. Paramagnetic beads were sourced from MagGenome, and AgNP (20 nm size, stabilized in citrate buffer, 20 mg ml^{-1}) were also obtained from Sigma-Aldrich. *Taq* polymerase and related reagents were acquired from Takara Clontech. The Twist Amp Liquid Basic RPA Kit came from TwistDX.

Cultures of *E. faecalis* (ATCC 29212), *S. aureus* (MTCC 96), *K. pneumoniae* (MTCC 109), *A. baumanii* (MTCC 12889), *P. aeruginosa* (MTCC 424), *Enterobacter* spp. (MTCC 7104), and *Neisseria gonorrhoeae* (ATCC 19424) were sourced from the American Type Culture Collection (ATCC, distributed by Himedia Laboratories Pvt. Ltd) or the Microbial Type Culture Collection and Gene Bank (MTCC) at the Institute of Microbial Technology (IMTECH), Chandigarh, India. The WHO BCG Danish 1331 vaccine sub-strain was obtained from the National Institute for Biological Standards and Control (NIBSC), UK.

Sample collection and DNA extraction

Genomic DNA was extracted from pure bacterial cultures using a standard kit-based method, specifically the DNEasy Blood and Tissue Kit (Qiagen) and NucleoSpin Tissue DNA, RNA and protein purification kit (Macherey Nagel), with minor adjustments to the manufacturer's protocol. For cost-effective and rapid gDNA isolation, we employed InstaDNA card method.[30–32] The resulting gDNA was quantified using NanoDrop Microvolume Spectrophotometers (Thermo Fisher Scientific) and utilized for amplification reactions with species-specific primers.

In the InstaDNA card-based DNA isolation method, a known bacterial culture of colony forming units (CFU), *e.g.*, 33×10^4 (in 2 μL volume), was blotted onto a 3 mm InstaDNA card disc, allowed to dry for 15–30 minutes at room temperature, washed in sterile water by just dipping the disc for 15 seconds, and transferred to the PCR reaction mix for amplification. Alternatively, the disc can be transferred to elution buffer (1× Tris–EDTA) for DNA elution by heating at 95 °C for 2–5 minutes. The eluent can further be used for RPA-based DNA amplification. Similarly, 10-fold serially diluted bacterial samples were blotted onto separate discs and processed accordingly in order to perform sensitivity assays using InstaDNA card followed by PCR and RPA, and various detection methods (gDNA dilutions and their corresponding bacterial number are provided in Table S2†).

In order to mimic the protein-rich environment of clinical samples, waste or leftover serum and urine from healthy subject was collected on InstaDNA card, air dried. We then punched out 3 mm discs using sterile punching machine. Each disc was then spiked with 20 ng gDNA of *K. pneumoniae*. The disc was then transferred to elution buffer (1× Tris–EDTA) for DNA elution by heating at 95 °C for 5 minutes. The eluent was estimated using NanoDrop Microvolume Spectrophotometers (Thermo Fischer Scientific), serially diluted and used for PCR and RPA-based DNA amplification.

PCR-AGE-based molecular detection

Specific oligonucleotide primers targeting *K. pneumoniae* were custom-designed and are detailed in Table 2. However, considerations during primer designing for RPA should include a higher GC content (≥50%), longer primer length (20–35 bp), and maintaining the amplicon size between 200-500 bp. Primers specific to KpSC strains were designed against *khe* gene (~404 bp) and *uge* gene (~222 bp). Primer specificity for all the gene targets was confirmed *in silico* through NCBI-BLAST analysis (Table S1†). Based on *in silico* analysis, primers against *khe* and *uge* are suitable for molecular detection of all the seven phylogroups of *K. pneumoniae*, however, current study has utilized the laboratory strain of *K. pneumoniae* (MTCC 109). PCR amplification using these primer sets was carried out in a Veriti Thermal Cycler from Applied Biosystems, with *Taq* Polymerase sourced from Takara Clontech, following the manufacturer's instructions with minor adjustments. Each 50 μl PCR reaction was prepared with a 1 μM primer mix in the reaction mixture, and thermal cycling included an initial denaturation step at 95 °C for 10 minutes, followed by 35 cycles of PCR conditions outlined in Table 2. The process concluded with a final extension at 72 °C for 10 minutes. The verification of PCR products was performed through AGE and DNA was visualized by ethidium bromide dye-based staining. The DNA products on the gel were quantified using ImageJ software (Version 1.8.0).

In order to validate the *in vitro* primer specificity for *K. pneumoniae*, a mix of gDNA samples obtained from diverse organisms which could possibly cause respiratory infections or pneumonia, were utilized as negative controls (NC) in PCR, such as *Mycobacterium tuberculosis* (*M. tb*), *Mycobacterium bovis* (*M. bovis*), *M. bovis* BCG, *N. gonorrhoeae*, *Chlamydia trachomatis*, *Ureaplasma urealyticum*, *E. faecalis*, *S. aureus*, *A. baumanii*, *P. aeruginosa*, *Enterobacter* spp. and human DNA. NC is prepared by mixing 5 ng gDNA obtained from each of these organisms, except *K. pneumoniae*. In order to determine interference in amplification due to presence of other pathogens or human host DNA (as anticipated in a clinical setting), we also included a control named, "Mix gDNA", which contains a mix of NC and genomic DNA of *K. pneumoniae*. This mix is prepared by mixing 5 ng gDNA of *K. pneumoniae* to the above-mentioned NC mix. For amplification reactions, 2 μl of NTC and Mix gDNA is used in case of PCR and RPA. For determination of detection sensitivity of PCR-AGE, serial ten-fold dilutions of gDNA templates of *K. pneumoniae* ranging from 20 nanograms, ng (3.3×10^6 bacteria) to 200 attogram, ag (3.3×10^{-2} bacteria) were employed in PCR reactions (Table S2†).

RPA-AGE-based molecular detection of *K. pneumoniae*

Isothermal nucleic acid amplification was performed for the pathogens using Twist Amp Liquid Basic RPA Kit (TwistDX, TALQBAS01 kit) as recommended by the manufacturer with

minor modifications. Briefly, all the reagents provided in the kit are mixed with defined quantity of DNA, reaction is initiated by addition of magnesium acetate, followed by incubation at 37 °C for 15–20 minutes. The RPA products are immediately cleaned up using XpressPure PCR Cleanup kit (MagGenome, MG20Pcr-50) as recommended by the manufacturer with slight modifications. Briefly, we used 0.8× volume/volume (v/v) of magnetic beads while purifying the RPA products and 1× v/v in case of PCR products. These modifications allowed elimination of smaller size primers and primer dimers from the amplification mix. Altogether, it offers simple, user-friendly, cost and time-efficient method for DNA purification without requiring any high-end electrical equipment. Similar to PCR-AGE assay, we determined the sensitivity or limit of detection of the RPA-AGE using serial ten-fold dilutions of gDNA templates ranging from (20 ng (3.3×10^6 bacteria)) to 200 ag (0.03 bacteria) (Table S2†). DNA was visualized by ethidium bromide dye-based staining of agarose gel.

Visual detection of amplified products using citrate-stabilized AgNPs

AgNP-based visual detection assay was performed using PCR amplified products as described by us previously,[34] wherein presence of DNA prevents the aggregation of AgNPs upon addition of aggregating agent, sodium chloride (NaCl) causing sliver dispersion to maintain yellow colour. However, in absence of DNA, addition of NaCl leads to AgNP aggregation causing grey coloration of the dispersion.

Briefly, 20 μl of RPA or PCR reaction products were made up to a volume of 50 μl in case of PCR and a volume of 100 μl in case of RPA using sterile water. Amplified products were then cleaned-up using MagGenome PCR clean up kit as per the manufacturer's instructions with slight modifications as mentioned earlier. Finally, DNA was eluted in 20 μl of elution buffer (10 mM Tris, 1 mM EDTA, pH 8.0) followed by incubation with 50 μl of the citrate-stabilized AgNP dispersion at room temperature for ~1 minute. Following this, 5 μl of aggregating reagent (5 M NaCl) was added to the assay tubes. The onset of AgNP aggregation is promptly revealed (in <1 min) by a grey colour dispersion in tubes lacking amplicons (negative controls with DNA from non-target organisms, NC or no-template controls, NTC). Conversely, in tubes with amplified DNA, the addition of NaCl prevents AgNP aggregation, resulting in the retention of a yellow colour. The entire process of purification and visual detection is completed within a time frame of 20–25 minutes. The sensitivity of detection was assessed by executing AgNP aggregation-based assay using amplicons derived from RPA conducted with varying amounts of gDNA isolated from *K. pneumoniae*.

Abbreviations

M. tb	*Mycobacterium tuberculosis*
M. bovis	*Mycobacterium bovis*
POC	point-of-care
ml	millilitre
μl	microlitre
ng	nanogram
pg	picogram
fg	femtogram
ag	attogram
M	molar
mM	millimolar
μM	micromolar
gDNA	genomic DNA
khe	*Klebsiella* hemolysin gene
uge	uridine diphospho (UDP)-D-galacturonate 4-epimerase gene
KpSC	*K. pneumoniae* species complex
PCR	polymerase chain reaction
DNA	deoxyribonucleic acid
qPCR	Quantitative PCR
RT-PCR	real time PCR
NTC	no-template-control
AGE	agarose gel electrophoresis
UV	ultraviolet
NaAc	sodium acetate
PEG	polyethylene glycol
AgNP	silver nanoparticles
Tris	tris(hydroxymethyl)aminomethane
EDTA	ethylene diamine tetra-acetic acid
NaCl	sodium chloride
LAMP	loop-mediated isothermal amplification
RPA	recombinase polymerase amplification
MCDA	multiple cross displacement amplification
BCG	Bacille Calmette-Guerin
WHO	World Health Organisation
CFU	colony forming unit
UTIs	urinary tract infections
LFS	lateral flow strip
HIV-1	human immunodeficiency virus-1
CRISPR	clustered regularly interspaced short palindromic repeats
v/v	volume/volume

Data availability

All data that supports the findings of this study are available from corresponding author upon reasonable request.

Author contributions

NP, NO and RJD have conceptualized the study. NP and NO have equally contributed to the work. NP and NO designed methodologies, curated data, performed analysis, designed figures for visualization of results. RJD and NP acquired the funding. NP, NO and RJD have written the original draft of the manuscript. All the authors reviewed and edited the manuscript.

Conflicts of interest

Authors declare no conflict of interest.

Acknowledgements

RJD is thankful to Birla Institute of Science and Technology (BITS) Pilani, Hyderabad campus, India for their funding support through intramural funding under Research Initiation Grant (RIG) and Centre for Human Disease Research (CHDR). RJD is thankful to Defence Research and Development Organization (DRDO), India for supporting through research grant (LSRB/81/48222/LSRB-367/BTB/2020). RJD is thankful to the overall infrastructure support by Department of Biological Sciences, BITS Pilani Hyderabad. NP is thankful to Indian Council of Medical Research (ICMR), Govt. of India for providing Senior Research Fellowship (RBMH/FW/2019/13). NO is thankful to LSRB, DRDO and BITS Pilani Hyderabad for fellowship. We acknowledge Dr Raghunand R Tirumalai, Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India for providing gDNA for *M. tuberculosis H37Rv*. Dr Bappaditya Dey, National Institute of Animal Biotechnology (NIAB), Hyderabad, India is acknowledged for *M. bovis* gDNA. We are grateful to Dr Benu Dhawan, Department of Microbiology, All India Institute of Medical Sciences (AIIMS, New Delhi, India) for giving gDNA from *Ureaplasma urealyticum*. We thank Dr Karthika Rajeeve, Rajiv Gandhi Centre for Biotechnology, Trivandrum, India for providing *Chlamydia trachomatis* (L2 serovar) gDNA.

References

1 V. Ballén, Y. Gabasa, C. Ratia, R. Ortega, M. Tejero and S. Soto, *Front. Cell. Infect. Microbiol.*, 2021, **11**, 738223.
2 M. K. Paczosa and J. Mecsas, *Microbiol. Mol. Biol. Rev.*, 2016, **80**, 629–661.
3 K. H. P. Riwu, M. H. Effendi, F. A. Rantam, A. R. Khairullah and A. Widodo, *Vet. World*, 2022, **15**, 2172.
4 E. Barbier, C. Rodrigues, G. Depret, V. Passet, L. Gal, P. Piveteau and S. Brisse, *Appl. Environ. Microbiol.*, 2020, **86**, e02711–e02719.
5 K. L. Wyres, M. M. Lam and K. E. Holt, *Nat. Rev. Microbiol.*, 2020, **18**, 344–359.
6 R. Podschun and U. Ullmann, *Clin. Microbiol. Rev.*, 1998, **11**, 589–603.
7 C. Y. Effah, T. Sun, S. Liu and Y. Wu, *Ann. Clin. Microbiol. Antimicrob.*, 2020, **19**, 1–9.
8 S. Navon-Venezia, K. Kondratyeva and A. Carattoli, *FEMS Microbiol. Rev.*, 2017, **41**, 252–275.
9 C. N. Murphy and S. Clegg, *Future Microbiol.*, 2012, **7**, 991–1002.
10 M. P. Bonasoni, A. Palicelli, G. Dalla Dea, G. Comitini, P. Nardini, L. Vizzini, G. Russello, M. Bardaro and E. Carretto, *Microorganisms*, 2021, **9**, 96.
11 J. P. Seliga-Siwecka and M. K. Kornacka, *Early Hum. Dev.*, 2013, **89**, 271–275.
12 K. Puri, D. H. Taft, N. Ambalavanan, K. R. Schibler, A. L. Morrow and S. G. Kallapur, *PLoS One*, 2016, **11**, e0162734.
13 K. Lindstedt, D. Buczek, T. Pedersen, E. Hjerde, N. Raffelsberger, Y. Suzuki, S. Brisse, K. Holt, Ø. Samuelsen and A. Sundsfjord, *Gut Microbes*, 2022, **14**, 2118500.
14 J. R. Bowers, D. Lemmer, J. W. Sahl, T. Pearson, E. M. Driebe, B. Wojack, M. A. Saubolle, D. M. Engelthaler and P. Keim, *J. Clin. Microbiol.*, 2016, **54**, 2582–2596.
15 K. D. Chavda, L. Chen, D. E. Fouts, G. Sutton, L. Brinkac, S. G. Jenkins, R. A. Bonomo, M. D. Adams and B. N. Kreiswirth, *mBio*, 2016, **7**(6), e02093.
16 Y. Liu, C. Liu, W. Zheng, X. Zhang, J. Yu, Q. Gao, Y. Hou and X. Huang, *Int. J. Food Microbiol.*, 2008, **125**, 230–235.
17 P. Kurupati, C. Chow, G. Kumarasinghe and C. L. Poh, *J. Clin. Microbiol.*, 2004, **42**, 1337–1340.
18 S. Ahmadi, H. Kamaladini, F. Haddadi and M. R. Sharifmoghadam, *J. Fluoresc.*, 2018, **28**, 987–998.
19 R. Nakano, A. Nakano, Y. Ishii, T. Ubagai, T. Kikuchi-Ueda, H. Kikuchi, S. Tansho-Nagakawa, G. Kamoshida, X. Mu and Y. Ono, *J. Infect. Chemother.*, 2015, **21**, 202–206.
20 X. Qiu, X. Liu, X. Ma, R. Wang, S. Chen, F. Li and Z. Li, *Microbiol. Spectrum*, 2022, **10**, e01545–e01522.
21 L. Niu, F. Zhao, J. Chen, J. Nong, C. Wang, J. Wang, N. Gao, X. Zhu, L. Wu and S. Hu, *PLoS One*, 2018, **13**, e0204332.
22 P. Hemwaranon, A. Srisrattakarn, A. Lulitanond, P. Tippayawat, R. Tavichakorntrakool, L. Wonglakorn, J. Daduang and A. Chanawong, *Antibiotics*, 2022, **11**, 1499.
23 N. Li, L. Wang, F. Wang, H. Chen, S. Tao, Q. Zhu, L. Liu, W. Liang and F. Ma, *Front. Cell. Infect. Microbiol.*, 2022, **12**, 877649.
24 B. Raja, H. J. Goux, A. Marapadaga, S. Rajagopalan, K. Kourentzi and R. C. Willson, *J. Appl. Microbiol.*, 2017, **123**, 544–555.
25 S. Lapa, S. Surzhikov, S. Blagodatskikh, V. Shershov and A. Chudinov, *Mol. Biol.*, 2023, **57**, 544–549.
26 R. K. Daher, G. Stewart, M. Boissinot and M. G. Bergeron, *Clin. Chem.*, 2016, **62**, 947–958.
27 Z. A. Crannell, B. Rohrman and R. Richards-Kortum, *PLoS One*, 2014, **9**, e112146.
28 Q. Guo, K. Zhou, C. Chen, Y. Yue, Z. Shang, K. Zhou, Z. Fu, J. Liu, J. Lin and C. Xia, *Front. Cell. Infect. Microbiol.*, 2021, **11**, 791997.
29 S. J. Priti, V. Baranwal, R. G. Dietzgen and A. Ghosh, *J. Pest Sci.*, 2021, **94**, 219–229.
30 R. Alyethodi, U. Singh, S. Kumar, R. Alex, G. Sengar, T. Raja, R. Deb and B. Prakash, *BMC Biotechnol.*, 2021, **21**, 36.
31 C. Ismailova, E. Golkocheva-Markova, T. Tenev and S. Krumova, *PROBLEMS of Infectious and Parasitic Diseases*, 2019, vol. 47, pp. 16–20.
32 S. Krumova, E. Golkocheva-Markova, S. Angelova, S. Voleva, A. Pavlova, I. Georgieva and P. Genova-Kalou, *Am. Sci. Res. J. Eng. Technol. Sci.*, 2019, **51**, 183–191.
33 G. da Cunha Santos, *Arch. Pathol. Lab. Med.*, 2018, **142**, 308–312.

34 N. Patnaik and R. J. Dey, *ACS Infect. Dis.*, 2023, **10**(2), 426–435.
35 C. Yin-Ching, S. Jer-Horng, L. Ching-Nan and C. Ming-Chung, *Microb. Pathog.*, 2002, **33**, 1–6.
36 L. J. Hartman, E. B. Selby, C. A. Whitehouse, S. R. Coyne, J. G. Jaissle, N. A. Twenhafel, R. L. Burke and D. A. Kulesh, *J. Mol. Diagn.*, 2009, **11**, 464–471.
37 M. Regué, B. Hita, N. Piqué, L. Izquierdo, S. Merino, S. Fresno, V. J. Benedí and J. M. Tomás, *Infect. Immun.*, 2004, **72**, 54–61.