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ORIGINAL ARTICLE

Recombinase polymerase amplification for fast, selective, DNA-based detection of faecal indicator *Escherichia coli*

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Significance and impact of the study: In this study, recombinase polymerase amplification (RPA) is presented as a fast, and highly selective method for the detection *Escherichia coli* DNA from diverse environmental strains. A novel RPA assay was compared with an existing, high performance qPCR, and demonstrated an equivalent inclusivity and specificity for the target species, with a significantly reduced analysis time. The RPA could be used to amplify and detect *E. coli* DNA in fewer than 3 min. The speed, selectivity and isothermal low temperature requirements of the RPA technique make it well-suited for on-site water quality testing.

Keywords
Escherichia coli, qPCR, RPA, water testing, isothermal.

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Abstract

The bacterium *Escherichia coli* is commonly associated with the presence of faecal contamination in environmental samples, and is therefore subject to statutory surveillance. This is normally done using a culture-based methodology, which can be slow and laborious. Nucleic acid amplification for the detection of *E. coli* DNA sequences is a significantly more rapid approach, suited for applications in the field such as a point of sample analysis, and to provide an early warning of contamination. An existing, high integrity qPCR method to detect the *E. coli ybbW* gene, which requires almost an hour to detect low quantities of the target, was compared with a novel, isothermal RPA method, targeting the same sequence but achieving the result within a few minutes. The RPA technique demonstrated equivalent inclusivity and selectivity, and was able to detect DNA extracted from 100% of 99 *E. coli* strains, and exclude 100% of 30 non-target bacterial species. The limit of detection of the RPA assay was at least 100 target sequence copies. The high speed and simple, isothermal amplification chemistry may indicate that RPA is a more suitable methodology for on-site *E. coli* monitoring than an existing qPCR technique.

Introduction

Water-borne pathogens remain a common and frequent cause of severe human and animal disease, worldwide (WHO 2019). The situation may be exacerbated by the increasing demands on global water resources, which must be met with new and efficient methods for the analysis of water microbiology to control public health risks. *Escherichia coli* is normally a commensal organism in the mammalian intestine, but it enters water resources in faeces, where it is considered as probable evidence of faecal contamination and the possible occurrence of enteric pathogens (Edberg *et al*. 2000; Odonkor and Ampofo 2013). It is, therefore, subject to statutory surveillance, for which the detection and enumeration of viable *E. coli* cells is normally done by recovering the organism from water samples and culturing them on selective and differential growth medium (SCA 2016). This requires a suitably equipped testing laboratory, meaning that samples are often transported off-site, and long incubation periods of more than 18 h are necessary before the results can be interpreted. Therefore, culture-based monitoring can be logistically and economically costly, and the delay means an increase in public health risk, especially during short-lived, stochastic contamination events.

Molecular biological methods, which use nucleic acid amplification to detect and count specific *E. coli* DNA or RNA sequences, could be used to address these limitations. They are culture-independent and generate relatively fast results; a typical DNA or RNA extraction and target sequence amplification and detection can be completed within a few hours (Mendes Silva and Domingues 2015). They are also relatively simple to automate (versus cell culture), and there are already portable DNA 'testers' enabling the analysis of samples on-site (Marx 2015). Other advantages include a greater inclusivity of diverse environmental strains, a very high selectivity for the target species and the ability to retest samples retrospectively for many years, once the genetic material has been isolated and suitably stored. Accordingly, nucleic acid amplification could complement existing culture-based laboratory analysis as a highly specific, advanced early warning system, suited to field use, and as a tool for the study of faecal indicator distribution and fate within water systems.

The 'gold standard' in nucleic acid amplification is the polymerase chain reaction (PCR) in which a DNA target sequence is almost exponentially copied by precisely controlling the reaction temperature. In 'cycles', a high temperature (>90°C) is applied to destabilize the DNA duplex and then a lower temperature is applied to promote the annealing and extension of oligonucleotide primers on a single-stranded target sequence by a heat-stable DNA polymerase. Sensitive and specific PCR-based detection of *E. coli* has been demonstrated by amplifying, for example, fragments of the genes *uidA* (Frahm and Obst 2003; Silkie *et al*. 2008), *tuf* (Maheux *et al*. 2011), *ybbW* (Walker *et al*. 2017; McQuillan and Wilson 2019) and *clpB* (McQuillan and Wilson 2019), and this has been demonstrated to have a better inclusivity and selectivity than culture (Walker *et al*. 2017). However, there are limitations. PCR requires precisely controlled, high temperatures which typically demand a stable and powerful energy source; an obstacle to the use of portable or deployable, battery operated field instruments. High temperatures cause other problems including the formation of bubbles and high pressure within reaction vessels, both of which are common issues affecting 'microfluidic' PCR devices. Additionally, the time taken to convert or 'ramp' between temperatures using conventional PCR machines means that a typical, full analysis can, presently, take more than an hour using modern instrumentation.

Isothermal nucleic acid amplification chemistries have become a popular alternative to PCR, in part because they do not require thermal cycling, and typically occur at lower temperatures (typically between 30 and 65°C) (Zanoli and Spoto 2012). For example, an isothermal nucleic acid sequence based amplification (NASBA) method for the direct amplification of *E. coli* mRNA requires a single 'primer annealing' step at 65°C followed by continuous amplification of the target sequence at 41°C (Min and Baeumner 2002; Heijnen and Medema 2009). Another employs the loop mediated amplification or LAMP technique for the amplification of *E. coli* DNA at a continuous 66°C (Hill *et al*. 2008). Other *E. coli* detection assays based on multiple displacement amplification (MDA) (Marcy *et al*. 2007) and helicase dependent amplification (HDA) (Mahalanabis *et al*. 2010) have similarly uncomplicated thermal requirements (versus PCR). However, although these methods obviate the need to continuously change the reaction temperature, they can still take in excess of an hour to generate a positive result, particularly when amplifying from low quantities of genetic material.

An emerging, isothermal amplification method is recombinase polymerase amplification (RPA). RPA was introduced in 2006, and has seen a significant increase in research applications (based upon the quantity of publications featuring the RPA technique), which may be due to its reported high speed and sensitivity. A recent, comprehensive review of the RPA technique highlights how RPA has been used to amplify DNA and RNA (by prior reverse transcription) from an array of bacterial, viral and metazoan target sequences, with examples of single cell sensitivity, and a positive result within a few minutes (Li *et al*. 2019). *Escherichia coil*-specific RPA has so far been limited to the detection of O157:H7 (Choi *et al*. 2016; Hu *et al*. 2020) using target DNA sequences that are not representative of general *E. coli* populations and, to the best of our knowledge, no such RPA method has been described that could be applied to faecal indicator *E. coli* testing.

This study was carried out to evaluate the RPA method for the selective, inclusive and rapid detection of general *E. coli* populations, towards a faster (vs existing PCR and isothermal assays) test for faecal indicator bacteria in environmental samples. An *E. coli*-specific RPA assay was developed to amplify a fragment of the *ybbW* gene, which was selected based on earlier work, and which identified this locus as highly conserved and specific to the target species (Walker *et al*. 2017). The assay included a target-specific, fluorometric 'exo' probe, for real-time detection of the amplified target. The selectivity, linearity and speed of the RPA method was evaluated using *E. coli* DNA extracted from a suite of laboratory and environmental strains.

Results and Discussion

In this study, a novel method for the detection and quantification of *E. coli* DNA was developed using RPA and commercially available RPA reagents, available from

TwistDx Ltd. The objective was to demonstrate RPA as a 'faster' alternative to an existing qPCR-based method, with equivalent performance in inclusivity of diverse *E. coli* environmental strains and selectivity for the target species. RPA primers and probe sequences were designed to anneal with a fragment of the *E. coli ybbW* gene coding sequence, a genetic locus which has already been determined to be both highly conserved within natural *E. coli* populations, and highly specific to this species (Walker *et al.* 2017; McQuillan and Wilson 2019). Multiple sequence alignment of *ybbW* gene sequences from diverse *E. coli* strains was employed to scrutinize the target sequence for potential oligonucleotide (primers and probe) annealing sites, as described in the materials and methods. Candidate primer sequences were screened for RPA activity using a specialized, target-specific fluorometric 'exo' probe together with a TwistAmp® Liquid exo Kit; a set of reagent solutions provided for the amplification and real-time measurement of target sequences using the proprietary TwistAmp® exo probe technology. Primers, which could be used to generate a detectable fluorescence within the shortest time, and the strongest fluorescence signal at the reaction end-point, were selected for further study. The primer and exo probe sequences used are given in Table 1.

TwistAmp Kit DNA inactivation

TwistAmp® RPA kits contain small amounts of *E. coli* DNA due to manufacturing methods. The presence and quantity of *E. coli* DNA in individual reagent solutions provided in the TwistAmp® Liquid exo kit was estimated using qPCR to amplify the *ybbW* target sequence, where present, from a sample of each provided solution. Positive amplification was observed for the 'Core Reaction Mix' (CRM) solution only; all other kit solutions contained undetectable levels of the target sequence. Amplification of the *ybbW* target sequence from the CRM in tandem with a series of *ybbW* sequence copy number standards was used to estimate that there were approximately 10^4 copies of the target sequence per microlitre of the CRM which, according to the reaction preparation method, would contribute approximately 12 500 copies to each reaction. The results were consistent between three different tests. To inactivate the DNA within the CRM, the reagent was exposed to 254 nm Ultraviolet (UV) radiation just prior to incorporation with the reaction mixtures; this was sufficient to eliminate detectable amplification from negative controls, without inactivating the CRM. However, UV radiation led to a modest reduction in the amplification efficiency (time until earliest detection) of the RPA reaction mixtures (Fig. S1).

Inclusivity and selectivity

The novel RPA assay was evaluated for both inclusivity and selectivity against a panel of genomic DNA samples, extracted from diverse *E. coli* strains and a range of non-*E. coli* bacterial species. For comparison, an existing *ybbW*-specific qPCR method, first described by Walker *et al.* (2017) and later refined (McQuillan and Wilson 2019), was tested in parallel. The results are shown in Table 2. The RPA method was able to detect 100% of 76 *E. coli* strains, including 72 strains belonging to the *E. coli* collection of reference (ECOR) strains, representing *E. coli* recovered from a range of different hosts and geographic locations (Patel *et al.* 2018). A total of three laboratory strains belonging to the K-12 lineage and a Type strain (NCTC 9001) were also detected by the RPA method, as well as 23 strains which had been isolated on selective and differential medium from contaminated dock water. In contrast, 100% of 30 non-*E. coli* species could not be detected (no detectable sequence amplification) by the RPA method, and these included closely related species including five additional members of the *Escherichia* genus and three members of the *Shigella* genus. The same selectivity results were obtained using the qPCR method, for which our results were in agreement with those reported in earlier work (Walker *et al.* 2017; McQuillan and Wilson 2019), further

Table 1 Oligonucleotides used in this study

Name	Type	Sequence (5′–3′)
ybbWPCRf	qPCR forward primer	TGATTGGCAAAATCTGGCCG
ybbWPCRr	qPCR reverse primer	GAAATCGCCCAAATCGCCAT
ybbWHP	qPCR Hydrolysis probe	[FITC]-CCGCCG[ZEN]AAAACGATATAGATGCACGG-[IABkFQ]
ybbWRPAf	RPA forward primer	TGCTTGATTCTGATTGGCAAAATCTGGCCG
ybbWRPAr	RPA reverse primer	GCCATACCGCCGAAAACGATATAGATGCACGGGTT
ybbWRPAexo	RPA exo probe	GTTTTAAATAAATTCACTGCCATTCTTAACCCG[FITCdT)G[THF]A[BHQ1dT]CTATATCGTTTTCG

FITC, Fluorescein Isothiocyanate; ZEN, ZEN internal fluorescence quencher; IABkFQ, Iowa Black Fluorescence Quencher; THF, Tetrahydrofuran; BHQ1, Black Hole Fluorescence Quencher-1.

confirming the *ybbW* target sequence as highly inclusive of genetic diversity in *E. coli*, and highly selective for this species.

Sensitivity, speed and linearity

The sensitivity, speed and linearity of the novel RPA assay were evaluated in tandem with the existing qPCR. This was done by using each method to amplify the target sequence from *E. coli* DNA copy number standards, prepared to contain between 10^7 copies and 1 copy of the *E. coli* genome. The RPA assay was found to respond to target sequence concentration over the range of 10^7–100 copies, with a simple linear regression finding a goodness of fit (R^2) to be 0·96. This is shown in Fig. 1a

The linearity of the response was weaker than that observed for the qPCR method ($R = 0{\cdot}99$), shown in Fig. 1b. The RPA method could be used to detect at least 100 copies of the *E. coli* genome, whereas the qPCR method could detect as few as 10 copies. However, UV irradiation of the TwistAmp® CRM reagent was necessary to inactivate unwanted *E. coli* DNA residue prior to RPA, and this procedure was found to reduce the RPA amplification rate. It cannot, therefore, be stated with any certainty that the limit of detection (LOD) of the assay is 100 copies. If alternative manufacturing processes were employed to prepare DNA-free RPA reagents, it is likely that the overall sensitivity and speed of the RPA method for *E. coli* would be improved. RPA detection of non-*E. coli* DNA sequences has, in many cases, been reported to demonstrate sensitivity to a single target sequence copy (Kalsi *et al.* 2015) or single cell (colony forming unit) (Ng *et al.* 2015; Kim and Lee 2016; Mondal *et al.* 2016; Ng *et al.* 2016), and it is reasonable to indicate that similar sensitivity could be achieved if the UV pre-treatment step could be avoided. Other, non-radiative, methods to eliminate DNA from the CRM reagent were considered in this work (data not shown), specifically endonuclease digestion, which may fragment the DNA contamination, and render it inactive in the amplification reaction. However, the subsequent elimination of the DNase activity using thermal denaturation also inactivated the CRM, even when using heat-labile enzymes which could be inactivated at 50°C.

Although the RPA method, in this case, was less sensitive than the qPCR, it was also significantly more rapid. For example, the selectivity testing, as described above, typically gave a positive result for *E. coli* DNA within 2 or 3 min albeit from a generous amount (approximately 1 ng per reaction) of DNA template. In comparison, the same DNA samples were amplified by qPCR, and at least 18 cycles (approximately 25 m 30 s) expired before a positive result could be interpreted. Using the DNA copy number standards, the RPA could be used to generate a positive result within 2 min (10^7 copies), taking no longer than 13 min (100 copies). Conversely, the qPCR technique required approximately 21·3 min (15 cycles) and 56·3 min (40 cycles) to generate a positive result from the same stock DNA samples. Using a modern thermocycling instrument such as the Roche LightCycler 96 (as used in this study), each PCR cycle requires 42 s to heat and cool the reaction. RPA is completed at a constant 37°C

Table 2 Selectivity and inclusivity of the RPA and qPCR assays

Species	Culture collection	*ybbW* RPA	*ybbW* qPCR
E. coli laboratory and environmental isolates (99)			
E. coli ECOR collection (strains 1-72)	STEC	+ (72)	+ (72)
23 Putative* *E. coli* Environmental Isolates	n/a	+ (23)	+ (23)
E. coli (type strain)	NCTC 9001	+	+
E. coli K12 (MG1655)	See note	+	+
E. coli K12 (W3110)	See note	+	+
E. coli K12 (DH5)	See note	+	+
Non *E. coli* bacteria (30)			
Escherichia fergusoni	NCTC 12128	–	–
Salmonella typhimurium	NCTC 1023	–	–
Vibrio cholerae	NCTC 8041	–	–
Shigella sonnei	DSM 5570	–	–
Shigella flexneri	DSM 4782	–	–
Escherichia albertii	DSM 17582	–	–
Shigella boydii	DSM 7532	–	–
Citrobacter freundii	DSM 30039	–	–
Escherichia vulneris	DSM 4564	–	–
Escherichia hermanii	DSM 4560	–	–
Salmonella bongorii	DSM 13772	–	–
Escherichia blattae	DSM 4481	–	–
Citrobacter koseri	DSM 4595	–	–
Pseudomonas aeroginosa	DSM 50071	–	–
Salmonella enterica (nottingham)	NCTC 7832	–	–
Aeromonas caviae	NCTC 10852	–	–
Klebsiella pneumoniae	DSM 30104	–	–
Pantoea agglomerans	NCTC 9381	–	–
Enterobacter aerongenes	NCTC 10006	–	–
Listeria monocytogenes	NCTC 11994	–	–
Enterococcus faecalis	NCTC 775	–	–
Enterococcus faecium	NCTC 7171	–	–
Lkluyvera cryocrescens	DSM 4588	–	–
Lelliottia amnigena	DSM 4486	–	–
Enterobacter cloacae	DSM 26481	–	–
Cronobacter sakazakii	DSM 4485	–	–
Klebsiella oxytoca	DSM 5175	–	–
Aeromonas hydrophila	DSM 30187	–	–
Rahnella aquatilis	DSM 4594	–	–
Providencia alcalifaciens	DSM 30120	–	–

Note: Some strains were selected from an in-house culture collection of laboratory *E. coli*.

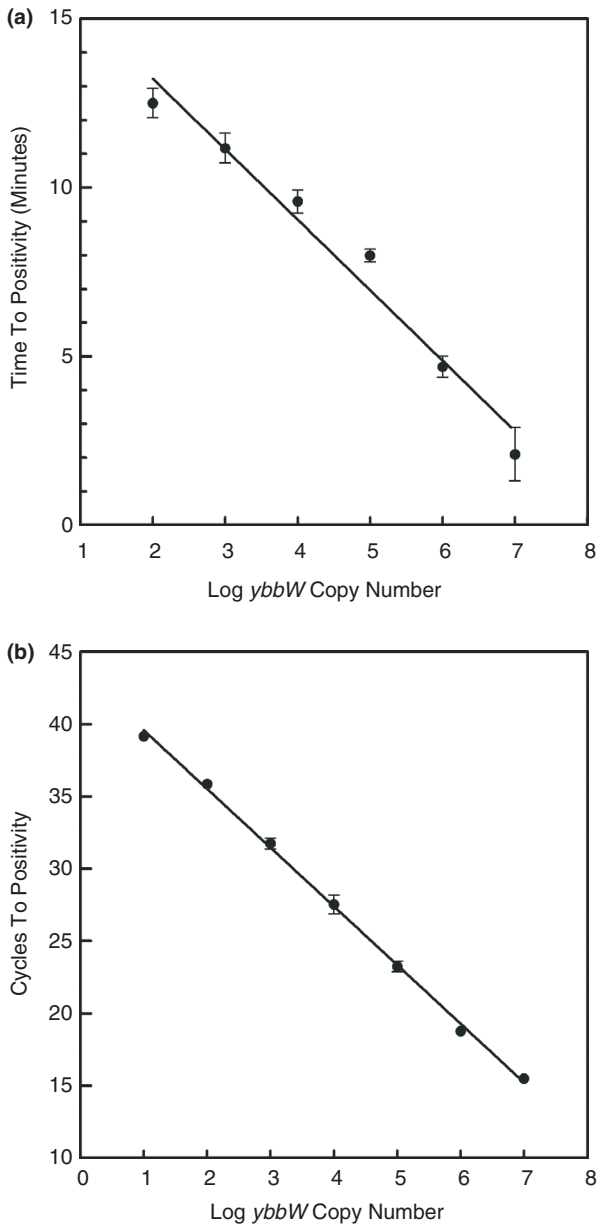

Figure 1 Time to positivity results for amplification of the *ybbW* target sequence using RPA (a) or qPCR (b). The *ybbW* target sequence was amplified using either the novel RPA method (a) or an existing qPCR method (b), which targeted the same genetic region in *Escherichia coli*. The error bars, where visible, represent the standard error of the mean from quadruplicate reactions.

without thermal cycling, such that the amplification occurred continuously throughout the incubation, and this contributed to the faster analysis time. Other, isothermal amplification techniques also obviate the thermal cycling requirement, however, may not occur as rapidly as RPA. For example, *E. coli* detection using isothermal NASBA required approximately 45 min to detect 100 copies of the target sequence (Walker *et al.* 2017), and isothermal LAMP can be used to positively detect *E. coli* in around 60 min (Hill *et al.* 2008). Therefore, our results suggest superior amplification reaction kinetics for the RPA technique, however, a direct comparison was not made during the course of this study.

The overall purpose of this study was to evaluate whether RPA could be used as a faster, isothermal alternative to qPCR for the detection and enumeration of faecal indicator *E. coli*. The RPA method had a short analysis time, requiring under 13 min to return a positive result from a sample containing 100 target sequence copies; the qPCR returned the same result in over 56 min. The speed of analysis for both methods is also dependent on, where required, the extraction and purification of DNA. Whilst many advances in molecular reagents have improved the efficiency of 'direct' analysis from crude sample preparations with little or no DNA purification, most applications will still require some form of sample processing. Nonetheless, even where a full DNA extraction is necessary, the whole procedure can still be completed within a fraction of the time required for culture. One other issue with molecular methods is the problem of discriminating live from dead cells using DNA, which can persist after cell inactivation, and this will also limit the application of molecular *E. coli* testing. One way to overcome this challenge is to measure mRNA, a more labile nucleic acid that degrades quickly after cell death. The RPA assay described in this work could easily be altered to target *ybbW* mRNA using Reverse Transcription RPA, however uncertain gene expression levels may compromise the quantitative nature of the assay or may exclude metabolically inactive cells. The use of DNA-binding dyes such a propidium monoazide (PMA) to inactivate DNA in dead cells prior to measurement could also be used to address this issue, based upon the integrity of the bacterial cell wall to discriminate living and dead cells (Nocker and Camper 2009).

The RPA assay demonstrated a sensitivity of 100 target sequence copies, which would normally correspond to 100 cells. It is likely that this would be improved without modification to the method, subject to the provision of DNA-free RPA reagents, but it was not possible to explore this within the scope of this work. Therefore, the current LOD for the method would limit its application to relatively high-level contamination events, for example sewerage overflows/leaks, or for the monitoring of wastewater discharge where higher levels of *E. coli* are expected. The routine surveillance of drinking and bathing water, for example, where the required sensitivity is a little as a single CFU per 100 ml of water, would require the use of more sensitive, culture-based methods. RPA detection of the target sequence over a wide

concentration range generated an approximately linear response, indicating its application as a quantitative assay, albeit the correlation was weaker than for the qPCR. The use of novel, RPA primer and probe sequences to detect *ybbW* had no discernible impact on the inclusivity or selectivity of the assay in comparison to the existing qPCR. The high speed of the analysis, coupled with the isothermal amplification reaction, would make this RPA assay better suited for use in fieldable, point of sample testing and, although the molecular methods in general are unlikely to replace culture-based techniques, their unique advantages have the potential to complement this approach for numerous *E. coli* surveillance applications.

Materials and Methods

Oligonucleotides

Oligonucleotide sequences used in this study are given in Table 1. All oligonucleotides were synthesized by LGC Biosearch Technologies (Denmark), and purified by high pressure liquid chromatography (HPLC). Oligonucleotides were delivered as dry, lyophilized residue which was hydrated in nuclease-free water at a concentration of 10 μmol l^{-1}, and stored at −20°C, in the dark.

Quantitative PCR

Quantitative PCR (qPCR) was carried out to determine the extent of *E. coli* contamination in commercially available RPA reagents and to compare the selectivity of qPCR and RPA oligonucleotide sets (Table 1) against a panel of bacterial DNA samples. All qPCR reactions were prepared using the GoTaq® G2 PCR System (Promega, Southampton, UK). Each reaction contained GoTaq® Colourless PCR Buffer at the manufacturer's recommended concentration: 1 mmol l^{-1} of $MgCl_2$, 0·5 mmol l^{-1} each of dATP, dTTP, dCTP and dGTP, 400 nmol l^{-1} of primers ybbWf and ybbWr, 200 nmol l^{-1} of hydrolysis probe ybbWHP, 1U of GoTaq® G2 polymerase and 1 μl of template DNA; the final volume was 20 μl. The reactions were prepared in 0.2 ml nuclease-free polycarbonate tubes with optically clear lids (Roche Diagnostics Ltd, Burgess Hill, UK). The reactions were completed using a LightCycler 96 real-time PCR instrument (Roche Molecular Systems Incorporated, Burgess Hill, UK), with an initial denaturation step of 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. The presence of *E. coli* contamination in RPA reagents was determined by preparing qPCR reactions to contain 1 μl of each reagent, and no additional DNA template. Enzyme-containing reagents were heated to 95°C for 5 min to inactivate the enzymes before testing, eliminating potential interference with the qPCR reactions. The number of *ybbW* sequences in each RPA reagent solution was estimated by comparing the C_t values of each reaction with those obtained from qPCR reactions containing 1 μl of a genomic DNA standard (10–10^7 copies of an *E. coli* genome). Standards were prepared from an *E. coli* type strain (National Collection of Type Cultures Strain 9001), exactly according to the method of Walker *et al* (Walker *et al*. 2017). All qPCR reactions were carried out in quadruplicate. The RPA reagent testing was repeated three times, using the reagents provided in three different TwistAmp® RPA kits (TwistDx Ltd, UK).

Assay design

A novel RPA assay for the detection of the *E. coli ybbW* gene sequence was designed using Geneious Version R11 (Biomatters Ltd, Auckland, New Zealand). Multiple sequence alignment of *E. coli ybbW* gene coding sequences from different *E. coli* isolates was completed using sequence information available from the National Centre for Biotechnology Information (NCBI) Genbank database. The alignment was used to identify suitable primer and probe annealing sites. Primer and probe sequences were selected with the aid of Primer 3 (Untergasser *et al*. 2012), and subject to a selectivity search using the Primer-BLAST algorithm (Ye *et al*.). In total, five forward primer, five reverse primer and two exo probe sequences were selected for study.

Recombinase polymerase amplification

RPA reactions were carried out using commercially available RPA reagent kits, provided in the TwistAmp® Liquid exo Kit, available from TwistDX Ltd (Cambridge, UK). The reactions were carried out according to the manufacturer's recommended protocol, and contained 400 nmol l^{-1} of each primer and 150 nmol l^{-1} of exo probe, 400 μmol l^{-1} of each dNTP; the final volume was 25 μl. The final volume included 1 μl of DNA template, which was either 1 ng of a bacterial DNA sample (for selectivity testing), or a DNA copy number standard of between 10^7 and 10 copies. The reactions were incubated at 37°C for 20 min. Real-time RPA reactions, incorporating a fluorescent exo probe (Table 1) were carried out using a LightCycler 96 real time PCR instrument, and real-time amplification curves were generated by measuring the fluorescence emission of Fluorescein Isothiocyanate (FITC) at 30-s intervals.

Inactivation of *E. coli* DNA in RPA reagents

RPA reaction mixtures were prepared as above, however, before the core reaction mix (CRM) reagent was added to

the reaction mixtures it was irradiated with UV light in order to degrade and inactivate any DNA contamination, which could cause a false-positive amplification. To do this, 10 μl of the CRM was dispensed into the cap of a 0·2 ml polycarbonate PCR tube, ensuring that it formed a discreet droplet in the centre of the cavity, and was not in contact with the walls. This was placed into a UV Crosslinker (Model UVP® C-1000, Fisher Scientific, Loughborough, UK) at a distance of precisely 15 mm from the UV source, and irradiated with 254 nm UV light for 102 s. The irradiated CRM was used immediately to prepare complete RPA reaction mixtures.

Selectivity Testing

The specificity and inclusivity of the RPA and qPCR methods described in this work was evaluated using a panel of genomic DNA samples isolated from different *E. coli* strains and non-*E. coli* bacteria. Genomic DNA was extracted from 1 ml of a broth culture of each strain in its optimal culture medium and incubation temperature (as per the recommendation of the relevant culture collection). The 'streak' plating method was used to confirm that each culture was pure. All culture media were purchased from Oxoid (Basingstoke, UK) Ltd. DNA was extracted using the GeneElute™ Bacterial Genomic DNA Isolation Kit (Sigma, Poole, UK), according to the manufacturer's recommendation, and stored at −20°C. The panel included the *E. coli* collection of reference strains (ECOR), laboratory strains of the K-12 lineage, a Type strain from the National Collection of Type Cultures (NCTC) and 30 non-*E. coli* strains purchased from various national and international culture collections (Table 2). Additionally, 23 strains of putative *E. coli* were recovered from the Empress Dock, Southampton between September and November 2019, and also tested. In this case, 100 ml of Dock Water was filtered onto a 0·45 micron pore size, 45mm diameter cellulose nitrate membrane disc (Fisher Scientific), which was placed directly onto TBX medium (Oxoid Ltd), and then incubated for 4 h at 30°C, followed by 18–24 h at 44°C. *Escherichia coli* were identified as blue/green colonies. These were picked with a sterile bacteriological loop, and used to inoculate 5 ml of Luria Broth culture, which was incubated at 37°C overnight. Then, 1 ml of the culture was used to prepare a DNA extract, using the method described above.

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. RPA fluorescence curves obtained when amplifying from the same DNA sample, with (dashed line) or without (solid line) the Ultra Violet (UV) pre-treatment to remove contaminating DNA from the Core Reaction Mix (CRM).