Pathology (December 2019) **51(7)**, pp. 733–736

MICROBIOLOGY

The design, validation and clinical verification of an in-house qualitative PCR to detect *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* in faeces

David Anthony Foley, Chor Ee Tan, Arleen Donaldson, Jade Vos, Samantha Hutton, Michelle N. D. Balm

Wellington Southern Community Laboratories, Wellington Hospital, Wellington, New Zealand

Summary

Yersiniosis is a zoonotic foodborne infection of public health significance. The aim of this study was to design and validate a simple, accurate and cost-effective polymerase chain reaction (PCR) to detect pathogenic *Yersinia* spp. in faecal samples.

An intercalating dye (EvaGreen)-based real-time multiplex PCR assay was designed to detect yadA, ystB and inv by melt curve analysis, allowing undifferentiated detection of all *Yersinia enterocolitica* biotypes, including biotype 1A, and *Yersinia pseudotuberculosis*. The assay was validated using cultured bacteria and clinical samples.

A total of 107 positive and 51 negative samples were tested. The sensitivity and specificity was 98% and 100%. The limit of detection was 10^4–10^5 CFU/g faeces. A total of 605 samples (9 positive) were tested in the clinical verification with an accuracy and negative predictive value of 99% [95% confidence interval (CI) 97.9–99.6%] and 99.8% (95% CI 97.9–99.6%), respectively.

This is an accurate, simple and cost-effective assay for the detection of pathogenic *Yersinia* spp.

Key words: Yersinia enterocolitica; YE; *Yersinia pseudotuberculosis*; YPT; PCR; faeces; design.

Received 1 January, revised 31 July, accepted 7 August 2019
Available online 10 October 2019

INTRODUCTION

Infectious gastroenteritis is a significant source of morbidity in New Zealand (NZ). There are an estimated 4.5 million episodes of acute gastrointestinal illnesses per year and a similar number of work days lost.[1] After *Campylobacter* spp. and *Salmonella* spp., yersiniosis is the third most common foodborne gastroenteritis in NZ, and is increasingly important in other parts of the world.[2,3] In 2017, the reported rate of yersiniosis in New Zealand was 19.7 per 100,000 individuals. Of the 834 isolates, 12 (1.4%) were identified as *Yersinia pseudotuberculosis* (YPT). The remainder were *Y. enterocolitica* (YE).[4] This frequency is more than nine times the rate of Australia and the European Union.[2,3] Underdiagnosis due to inadequate testing is a problem. The true incidence is estimated to be almost 20 times this figure.[2]

YE is composed of diverse subtypes, separated through biochemical reactions and serotyping of the lipopolysaccharide O antigens.[5] In New Zealand, YE biotype 2/3 are the most frequently isolated (68%), with biotype 1A (22%) and 4 (11%) occurring less commonly.[6] Biotype 1A strains are traditionally considered non-pathogenic.[5] However, there is clinical, epidemiological and experimental evidence that strains of biotype 1A, especially those isolated from faecal specimens during a gastrointestinal illness, are pathogenic.[7] Although biotype 1A can lack the pYV plasmid and chromosomal genes, ystA and myfA, alternative virulence factors may be present including the heat stable toxin ystB and hreP.[8] It has also been suggested that that YE biotype 1A can be further genetically subtyped into pathogenic and non-pathogenic strains.[9]

Available commercial faecal polymerase chain reaction (PCR) panels at present do not include relevant *Yersinia* targets, making their widespread introduction problematic both in NZ and worldwide. This report proposes a simple, accurate and cost-effective real time PCR designed to work in tandem with pre-existing work flow utilising a commercial panel.

MATERIAL AND METHODS

Triplex qualitative PCR design and optimisation

The primers sets (Supplementary Table 1, Appendix A) were selected from the literature[10,11] and specificity confirmed by NCBI BLASTn[12] search. The primer sets were chosen for their compatibility and specificity for pathogenic YE, including biotype 1A, and YPT. The yadA and inv targets can also be present in *Y. pestis*, a pathogen not present in our region. Primer concentration, annealing temperature and extension time were optimised as singleplex assays before being combined as a triplex.

DNA extraction

Faecal and culture strains were suspended in STAR buffer (Roche Diagnostics, France) and stored at −80°C prior to DNA extraction. DNA was extracted on the MagNA Pure 96 using the DNA and Viral NA Small Volume Kit (Roche Diagnostics) according to the recommended protocol.

The *Yersinia* biotypes used for optimisation and strain types for positive controls are outlined in Table 1. The reaction was performed in a final volume of 20 µL PCR reactions containing 5 µL of DNA extract, 4 µL EvaGreen qPCR mastermix (Biotium, USA), 8.6 µL PCR grade water, 0.4 µL of 10 µM of each forward and reverse primer. Intercalating dye chemistry (EvaGreen; Biotium) was selected to minimise assay costs. qPCR was performed using a real time PCR cycler system (Light Cycler 480 system; Roche Diagnostics)

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: https://doi.org/10.1016/j.pathol.2019.08.001

Table 1 Control organism, limit of detection (LOD) and efficiency by primer set

Target	Control organism	Culture-LOD	PCR-LOD	Slope	Efficiency
yadA	YE Biotype 4 NZRM 3596	10^2 CFU/g	10^4 CFU/g	−3.360	1.984
ystB	YE Biotype 1A 15ER0056	10^2 CFU/g	10^5 CFU/g	−3.525	1.922
Inv	YPT NZRM 768	10^2 CFU/g	10^5 CFU/g	−3.433	1.955

using a protocol of initial denaturation at 95°C for 12 min, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 63°C for 16 s, with a step down of 0.25°C per cycle to a secondary target of 60°C. Melt curve analysis was performed on a range from 78°C to 95°C with 25 acquisitions per °C. Results were interpreted as per Supplementary Table 2 (Appendix A).

Triplex dynamic range, PCR efficiency and limit of detection (LOD)

The dynamic range could not be calculated as there was late cycle threshold (CT) non-specific rise in fluorescence secondary to the matrix composition. The methodology for analytical sensitivity was adapted from the report by Mokhtari *et al.*[13] Faeces were suspended in 10% weight per volume PBS. A 0.5 McFarland concentration was made from overnight culture of the test organism (control strains); 1:10 serial dilutions were made and 100 µL of each was inoculated onto 5% sheep blood agar. Plates with more than 50 and less than 300 colonies were counted to calculate actual colony forming units (CFU) per 1000 µL; 100 µL of each dilution was added to 900 µL of faeces to achieve a 10% weight per volume concentration. This was vortexed for 5 s and a 10 µL loop was inoculated on *Yersinia* selective agar and into 1000 µL of STAR buffer. The *Yersinia* selective agar was incubated at 28°C and read at 24 and 48 h. Colonies with typical morphology were identified by mass spectrometry matrix-assisted laser desorption/ionisation-time of flight (MS MALDI-TOF). DNA was extracted as per the previous outlined method. The PCR efficiency was calculated using a series of four $\log_{10}$ dilutions of the extracted control organism run in triplicate (see Table 1).

Assay validation

Bacterial strains

A panel of 158 samples that included 98 faecal extracts and 60 direct colony extracts was used for validation (Table 2). Faecal extracts included YE, YPT and six other *Yersinia* spp. including *Y. massiliensis, Y. bercovieri, Y. frederiksenii* and *Y. intermedia*. The *Yersinia* culture negative samples in the faecal extracts included *Salmonella* spp. (*n*=2), *Campylobacter* spp. (*n*=3), *Shigella* spp. (*n*=3) and *E. coli* O157 (*n*=1). The 60 direct colony isolates obtained from the reference laboratory included YE, YPT and *Yersinia massiliensis*. The direct colony extracts were tested by blinded assessment.

Clinical verification

Clinical samples were tested over a period of 21 days. The triplex *Yersinia* qPCR was compared to culture. Faeces (10 µL) was inoculated on to *Yersinia* isolation agar (Fort Richard, New Zealand) and incubated at 28°C. The plates were read at 24 and 48 h. Colonies with typical morphology were initially identified by Biomerieux MS MALDI-TOF (France) and confirmed, including bio-typing, by the local reference laboratory.

RESULTS

Figure 1 shows a typical melting peak of the triplex *Yersinia* PCR assay. Non-specific melting peaks can be observed at 79°C. The melting peak of each target is identified with its corresponding melting temperature.

Table 2 Test organism by matrix and detection rate

Organism	Faeces		Direct colony		Total
	N	Detected (%)	N	Detected (%)	Detected (%)
YE	48	46 (95.8%)	43	43 (100%)	89 (97.8%)
Biotype					
1A	17	15 (88.2%)	10	10 (100%)	25 (92.3%)
2/3	26	26 (100%)	23	23 (100%)	49 (100%)
4	5	5 (100%)	10	10 (100%)	15 (100%)
YPT	6	6 (100%)	10	10 (100%)	16 (100%)
Other *Yersinia* spp.	6	0	7	0	0
Culture negative faecal samples	38	0	–	–	0

YE, *Yersinia enterocolitica*; YPT, *Yersinia pseudotuberculosis.*

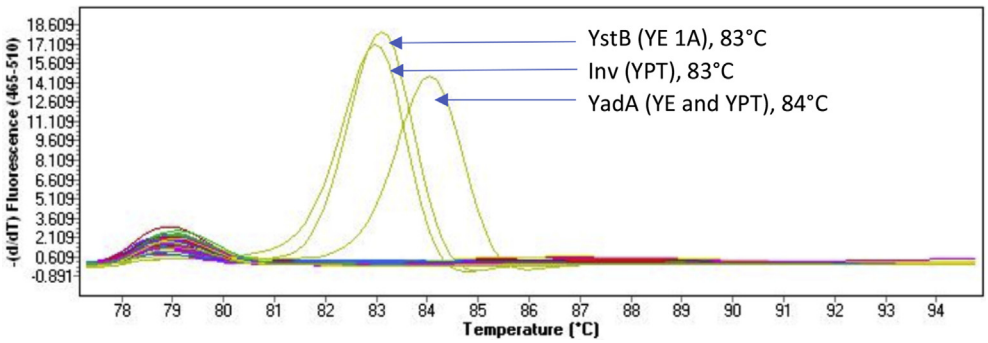

Fig. 1 Example of a Yersinia PCR Tm calling result from the LightCycler 480 II.

The analytical specificity and clinical sensitivity was assessed using the samples outlined in Table 2. This included both pathogenic YE and YPT from faeces and direct colonies. Two culture positive isolates typed as YE biotype 1A were not detected by the assay. The combined sensitivity, specificity and accuracy are outlined in Table 3.

Measurement of uncertainty

The inter-run variation of the melting temperature was calculated using the Tm values of the positive controls over five runs as given. The intra-run variation of the melting temperatures for each gene target was calculated using the Tm of known positives (Table 4).

Clinical verification results

A total of 605 clinical samples were tested. Of these, four were culture positive and 601 were culture negative. Eight samples were PCR positive. One biotype 1A initially tested as negative was detected on repeat testing, with the target present at the LOD. Five other samples that were culture negative were PCR positive (Table 5). These samples were re-tested by multiplex and by single target for each assay. The target amplicon, yadA, was sequenced using the assay primers, with 100% homology when BLAST was performed. The assay demonstrated a clinical specificity of 100% when these samples were counted as true positives.

DISCUSSION

This report outlines the design, validation and clinical verification of a qualitative PCR to detect YE and YPT, both of which are important enteropathogens. Their rapid and reliable detection may improve patient care and reduce unnecessary interventions.[14,15] As more faecal pathogen panels using nucleic acid amplification techniques are adopted, it is important to know the limitations of the selected targets. This has become more difficult with the widespread adoption of commercial panels with restricted proprietary information.

The three targets utilised in this multiplex demonstrated high clinical and analytical sensitivity and specificity. It allowed the pan detection of YE and YPT, including the majority of the more difficult to detect biotype 1A. ystB is a major contributor to diarrhoea.[16] YE biotype 1A without ystB are considered markedly less virulent.[9] We assert that detecting ystB target is an important step in identifying pathogenic strains of YE biotype 1A. This multiplex did not detect two YE biotype 1A in clinical samples in the validation component. These samples remained negative on repeat testing. This may be due to sampling bias, CFU below detection limit or, importantly, absence of the ystB

Table 3 Triplex combined sample sensitivity, specificity and accuracy

	Total positive	Total negative	
PCR positive	105	0	PPV 100% (95% CI 100.00%)
PCR negative	2	51	NPV 96.2% (95% CI 86.6–99%)
Total	107	51	Accuracy 98.8% (95% CI 95.5–99.9%)
	Sensitivity 98% (95% CI 93.4–99.8%)	Specificity 100% (95% CI 93–100%)	

CI, confidence interval; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value.

Table 4 Inter- and intra-run variation

Target	Inter-run variation			Intra-run variation		
	N	Mean Tm °C (range)	Coefficient variance	N	Mean Tm °C (range)	Coefficient variance
yadA	5	83.99°C (83.1–84.36)	0.26	32	84.01°C (83.75–84.18)	0.1
ystB	5	83.49°C (82.93–84.02)	0.33	10	83.39°C (82.93–83.62)	0.27
inv	5	83.3°C (82.95–83.82)	0.22	10	83.31°C (83.15–83.53)	0.16

Table 5 Triplex combined clinical verification sensitivity, specificity and accuracy

	Culture positive	Culture negative	
PCR positive	3	5[a]	PPV 37.5%[a] (95% CI 17.5–63%)
PCR negative	1	596	NPV 99.8% (95% CI 99–100%)
Total	4	601	Accuracy 99% (95% CI 97.9–99.6%)
	Sensitivity 75% (95% CI 19.4–99.4%)	Specificity 99.2% (95% CI 98.1–99.73%)	

Specificity was confirmed as outlined in the text.
CI, confidence interval; NPV, negative predictive value; PCR, polymerase chain reaction; PPV, positive predictive value.
[a] Five samples were PCR positive, culture negative.

chromosomal target. A further YE biotype 1A was not detected in the verification step that was detected on repeat testing.

In the post validation assessment, five PCR positive specimens were culture negative. The amplicons were sequenced and BLAST performed confirming the presence of yadA. One explanation is loss of viability due to delay in culture, however *Yersinia* spp. are relatively robust and can thrive at lower temperatures. Other potential explanations include isolates not having the typical morphology, not being recognised by laboratory scientist or being overgrown by a commensal organism. A likely explanation is that the presence of multiple copies of yadA improved the sensitivity of PCR. yadA has a lower LOD as it is plasmid target with the potential for multiple copies per cell. The LOD for culture and LOD in CFU/g for yadA was consistent with previous reports.[17] The LOD for ystB and inv were one log lower. The significance of this LOD is questionable given the detection rate from clinical samples in this report. Enrichment prior to culture or PCR can improve LOD. However, this is more labour intensive and would diminish the time-saving benefits of PCR.[18]

We developed this in-house assay for the detection of pathogenic Yersinia strains not available at the time in faecal pathogen molecular panels, thus avoiding the need for parallel culture. The combination of targets utilised provides a cost effective and specific assay for detection of recognised pathogenic *Yersinia* spp. Given the requirement to combine targets to ensure accuracy, it is expected that this assay would out-perform the currently available commercial assays. However, this cannot be confirmed without knowing the commercial targets utilised or a head-to-head assessment which is outside the scope of this report. Overall, the assay has improved workflow in our laboratory, providing improved turnaround times and capacity. Stool culture is reserved for PCR positive samples for epidemiological and antimicrobial resistance surveillance purposes, allowing more efficient workflows and resource use. Culture, in turn, ensures the specificity of the assay, but specialist interpretation of PCR positive, culture negative samples is required, especially given the use of a plasmid target.

CONCLUSION

This assay is an easy to use, cost-effective and efficient triplex with high sensitivity and specificity for the detection of clinically relevant *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* strains. It can be used as a stand-alone assay or to complement a commercial panel PCR for enteric pathogens in areas where yersiniosis is an important cause of gastroenteritis.

Acknowledgements: We would like to acknowledge Jackie Wright and the Institute of Environmental Science and Research Ltd for supplying a blinded test panel of samples for assay validation.

Conflicts of interest and sources of funding: The authors state that there are no conflicts of interest to disclose.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2019.08.001.

Address for correspondence: Dr David Foley, Wellington Southern Community Laboratories, Level 5 CSB, Wellington Hospital, Riddiford St, Newtown, Wellington, 6021, New Zealand. E-mail: drdavidanthonyfoley@gmail.com

References

1. Adlam SB, Perera S, Lake RJ, *et al*. Acute gastrointestinal illness in New Zealand: a community study. *Epidemiol Infect* 2011; 139: 302–8.
2. Gadiel D, Abelson P. *The Economic Cost of Foodborne Disease in New Zealand*. Wellington: New Zealand Food Safety Authority, 2010.
3. European Centre for Disease Prevention and Control. *Yersiniosis. Annual Epidemiological Report for 2016*. Stockholm: ECDC, 2018. https://ecdc.europa.eu/sites/portal/files/documents/AER_for_2016-yersiniosis.pdf
4. Institute of Environmental Science and Research. *New Zealand Public Health Surveillance Report* Mar 2018; 16 (1); cited Dec 2018. https://surv.esr.cri.nz/PDF_surveillance/NZPHSR/2018/NZPHSRMarch2018.pdf
5. Petsios S, Fredriksson-Ahomaa M, Sakkas H, *et al*. Conventional and molecular methods used in the detection and subtyping of Yersinia enterocolitica in food. *Int J Food Microbiol* 2016; 237: 55–72.
6. Galindo CL, Rosenzweig JA, Kirtley ML, *et al*. Pathogenesis of Y. enterocolitica and Y. pseudotuberculosis in human Yersiniosis. *J Pathog* 2011; 2011: 182051.
7. Tennant SM, Grant TH, Robins-Browne RM. Pathogenicity of Yersinia enterocolitica biotype 1A. *FEMS Immunol Med Microbiol* 2003; 38: 127–37.
8. Peruzy MF, Murru N, Perugini AG, *et al*. Evaluation of virulence genes in Yersinia enterocolitica strains using SYBR Green real-time PCR. *Food Microbiol* 2017; 65: 231–5.
9. Sabina Y, Rahman A, Ray RC, *et al*. Yersinia enterocolitica: mode of transmission, molecular insights of virulence, and pathogenesis of infection. *J Pathog* 2011; 2011: 429069.
10. Fukushima H, Kawase J, Etoh Y, *et al*. Simultaneous screening of 24 target genes of foodborne pathogens in 35 foodborne outbreaks using multiplex real-time SYBR green PCR analysis. *Int J Microbiol* 2010; 2010: 864817.
11. Thoerner P, Bin Kingombe CI, Bogli-Stuber K, *et al*. PCR detection of virulence genes in Yersinia enterocolitica and Yersinia pseudotuberculosis and investigation of virulence gene distribution. *Appl Environ Microbiol* 2003; 69: 1810–6.
12. Boratyn GM, Schaffer AA, Agarwala R, *et al*. Domain enhanced lookup time accelerated BLAST. *Biol Direct* 2012; 7: 12.
13. Mokhtari W, Nsaibia S, Gharbi A, *et al*. Real-time PCR using SYBR Green for the detection of Shigella spp. in food and stool samples. *Mol Cell Probes* 2013; 27: 53–9.
14. Zinczuk J, Wojskowicz P, Kisluk J, *et al*. Mesenteric lymphadenitis caused by Yersinia enterocolitica. *Prz Gastroenterol* 2015; 10: 118–21.
15. Laji N, Bowyer R, Jeyaratnam D, *et al*. Another mistaken case of appendicitis. *BMJ Case Rep* 2015; 2015; bcr2015211861.
16. Singh I, Virdi JS. Production of Yersinia stable toxin (YST) and distribution of yst genes in biotype 1A strains of Yersinia enterocolitica. *J Med Microbiol* 2004; 53: 1065–8.
17. Ganji L, Azimirad M, Farzi N, *et al*. Comparison of the detection limits of the culture and PCR methods for the detection of Clostridium difficile, Clostridium perfringens, Campylobacter jejuni, and Yersinia enterocolitica in human stool. *Arch Pediatr Infect Dis* 2016; 5: e38888.
18. Al-Talib H, Latif B, Mohd-Zain Z. Pentaplex PCR assay for detection of hemorrhagic bacteria from stool samples. *J Clin Microbiol* 2014; 52: 3244–9.