DETECTION AND ENUMERATION OF *YERSINIA ENTEROCOLITICA* FROM RAW PORK: PILOT SURVEY

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Nicola King
Dr Andrew Hudson

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Institute of Environmental Science & Research Limited
Christchurch Science Centre
Location address: 27 Creyke Road, Ilam, Christchurch
Postal address: P O Box 29 181, Christchurch, New Zealand
Website: www.esr.cri.nz

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Dr Stephen On
Food Safety Programme Manager

Dr Andrew Hudson
Project Leader

Dr TeckLok Wong
Peer Reviewer

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

SUMMARY

In 2005, the incidence of yersiniosis in New Zealand was 10.9 cases per 100,000 people and was the third most notified foodborne enteric pathogen (ESR, 2006). Most human infections are caused by a small number of pathogenic biovars and serotypes of *Yersinia enterocolitica* carrying the virulence plasmid pYV, and these pathogens are isolated more commonly from pork than any other food derived from animals. There is only very limited data on the prevalence and concentration of *Y. enterocolitica* in the New Zealand food supply, mostly because this bacterium is difficult to isolate from more competitive background microflora and is often present at very low numbers. This document reports on a recent survey for pYV-positive *Y. enterocolitica* (*Ye*P+) on retail raw pork using a new rapid and sensitive method developed at ESR to both detect (presence/absence) and enumerate (by most probable number method) this pathogen.

Forty-one raw pork samples from Christchurch supermarkets and butcher's shops were tested by the presence/absence (P/A) method only, by MPN (3×3) only, or simultaneously by both methods. Whole meats (8 by P/A, 9 by MPN, 17 both) were swabbed and part-processed meats (2 by P/A, 2 by MPN, 17 both; mostly mince) were rinsed in broth to extract any *Ye*P+. A robust PCR analysis was combined with selective enrichment and plating media to detect and isolate *Ye*P+, which were further confirmed by biochemical tests and PCR. A presumptive P/A result is obtained within 24 hours of sample receipt, and any presumptive *Ye*P+ isolates confirmed within four days.

Of the 41 samples tested for the presence of *Ye*P+ during this survey, 42% were presumptively positive (PCR-positive) and 22% yielded *Ye*P+ isolates. Under P/A testing, six of the 25 whole meats and four of the five minces were presumptively positive by PCR, and isolates were confirmed from three of the whole meats and three of the mince samples. Under MPN testing, seven of the 26 whole meats and four (all mince) of the five part-processed meats tested were PCR-positive for *Ye*P+ in at least one MPN tube, and isolates were confirmed from five of the whole meats and two of the mince samples.

The PCR detection was possibly more robust than the cultural methods (PCR-positive samples did not always yield a *Ye*P+ isolate). Making the assumption that none of the PCR-positive samples represent false positive results, MPN values were best calculated from PCR results, provided at least one *Ye*P+ isolate was obtained from any of the MPN tubes for a sample. Of the 11 samples that were PCR-positive in the MPN testing, MPN values could be calculated for only six, of which two samples did not yield *Ye*P+ isolates. For the six PCR-positive samples where an MPN value could be calculated, the concentration of *Ye*P+ in the three whole meats was 0.30, 1.52 and 5.42 MPN/cm^2, was 0.31 and 2.48 MPN/g in two of the mince samples, and was >42.90 MPN/g in the third mince sample.

Using spiked samples, a conservative detection limit for presence/absence analysis can be approximated to 10 cfu/cm^2 for whole meats, and 100 cfu/g for minced meat. The analysis of spiked samples by MPN demonstrated that, while the detection limit was equivalent for presence/absence, there were major discrepancies between the MPN result and the known spiked concentration on three occasions. There is a need to improve the detection limit of the MPN system, but the strategy will depend on the objectives of any analytical work.

1 INTRODUCTION

Yersinia enterocolitica has been isolated from humans with acute enteritis, and is an important foodborne pathogen in New Zealand. Over the last five years the number of yersiniosis notifications in New Zealand was consistently between 400 and 500 cases per year (NZPHO, 2006). In 2005, the annual incidence was 10.9 cases per 100,000 people; the third most notified foodborne enteric pathogen (ESR, 2006). The average number of cases per month during 2005 was 34, and while the highest number of cases was associated with cities, the highest rates were reported in the West Coast, Wellington and Wanganui (NZPHO, 2006).

Y. enterocolitica can be isolated from many foods of animal origin but most are non-pathogenic strains. Most human infections are caused by a small number of pathogenic serotypes and biovars (Barton & Robins-Brown, 2003). It is common for fully virulent strains of *Y. enterocolitica* to carry the 'plasmid for *Yersinia* virulence', pYV, which encodes a number of virulence factors (Robins-Browne, 1997). Serotype O:3 is most frequently isolated from humans worldwide, and almost all are biovar 4. Serotypes common to the European region include O:9 and O:5, and O:8 is common in North America. Almost 90% of New Zealand cases are serotype O:3 (Hudson *et. al*., 2001; Robins-Brown, 1997). Serotypes of *Y. enterocolitica* most commonly associated with human disease are more frequently isolated from pigs than other food animals. However, with the exception of pork tongue, the bacterium has been rarely isolated from retail pork meat, though the standard methods for *Yersinia* isolation from food might account for this low detection (Robins-Brown, 1997).

Data on the prevalence and concentration of *Y. enterocolitica* in the New Zealand food supply is limited. The main reason is the lack of a rapid and reliable method to isolate and enumerate *Y. enterocolitica*. Isolation of pathogenic *Y. enterocolitica* from foods is confounded by the likelihood that the bacterium is present in small numbers and requires enrichment for detection, and by the presence of high numbers of competitive background microflora and non-pathogenic *Yersinia* species. Recent methods for isolation and identification rely on selective enrichment procedures, specific biochemical reactions and molecular techniques. ESR has developed a system that brings together techniques and specific media from a number of publications to analyse meat samples for the presence of low numbers of pYV-positive *Y. enterocolitica* (henceforth abbreviated to *Ye*P+), and to enumerate using a 'most probable number' (MPN) method. The presence/absence and MPN methods were trialled on retail raw pig meats sampled from outlets in Christchurch. The results of this pilot survey are reported in this document. Full details of the method development will be submitted as a draft paper in August 2006.

2 SURVEY METHODOLOGY

2.1 Sampling programme

Raw pork samples were purchased from four supermarkets and three butcher's shops in Christchurch. The supermarkets were visited on at least two occasions over the sample period (May and June, 2006), and the butcher's shops once. The samples selected depended on what was available at time of purchase, and were a mixture of pre-packaged meats displayed in open refrigerators and meats from delicatessens. If a range of packaging dates was available, an attempt was made to vary the age of the meat purchased. Every sample purchased differed with regard to cut, location or packaging date.

A total of 41 samples were purchased. Of these, seven were part-processed meats (6 mince, 1 stir-fry cut) and the remainder were a mixture of schnitzel, chops and steak cuts (called whole meats). Twenty of these samples (17 whole meats, 3 mince samples) were analysed simultaneously by presence/absence (P/A) testing and by MPN (Table 1), so a total of 61 analyses were performed. All samples were chilled as quickly as possible after purchase and analysed within 24 hours.

Table 1: Samples of retail raw pork analysed during the pilot survey

Meat samples	**Analysis**			
	P/A	MPN	Both*	*Total*
Whole meats	8	9	17	*34*
Part-processed meats	2	2	3	*7*
Total	*10*	*11*	*20*	*41*

* Analysed by both P/A and MPN methods

2.2 Preparation of meats

2.2.1 Whole meats

For each sample, a piece of meat was randomly selected from the packet and a square measuring approximately 25 cm^2 was aseptically excised. The square was placed in a sterile petri dish and refrigerated until analysed.

2.2.2 Part-processed meat

For each sample, 5 g was weighed into a sterile filter bag (actual weight recorded) and 50 ml Ossmer broth added. The sample was homogenised in a stomacher for 1 min and the coarsely-filtered liquid poured off into a 50 ml centrifuge tube. Processing continued as detailed below.

2.2.3 Spiked samples

For every set of samples analysed, at least one positive (spiked) meat sample was prepared and simultaneously analysed. An overnight culture (5 ml trypticase soy broth (TSB), 24ºC) of *Y. enterocolitica* NZRM 3596 (O:3, pYV-positive) was used for all spiked samples, and for positive controls. The culture was serially diluted in 0.1 % peptone and variable dilutions were selected for spiking over the course of the survey. For whole meats, 50 µl from the selected dilution was spread over the upper surface of one 25 cm^2 square from a randomly selected sample. This was refrigerated for at least 40 min before analysis to allow for attachment. For part-processed meats, 50 µl of a selected dilution was inoculated into a broth/meat mix prior to homogenisation. The inoculum was calculated by the preparation of trypticase soy agar plate counts, which were incubated at 37ºC, overnight.

2.3 Analysis of samples for the presence or absence of pYV-positive *Y. enterocolitica*

2.3.1 Preparation of enrichments

2.3.1.1 Whole meats

For each sample, the entire upper surface was swabbed with a cotton-tipped swab moistened with potassium hydroxide (KOH) solution (0.25%:0.5% KOH:NaCl), followed with a dry swab. Both swabs were broken into a centrifuge tube containing 9 ml Ossmer broth, shaken vigorously, and then incubated at 24ºC for 18 h. A positive control consisting of 9 ml Ossmer broth inoculated with 100 µl of an overnight culture (5 ml TSB, 24ºC) of *Y. enterocolitica* was also prepared and incubated under the same conditions, along with a negative (sterility) control (9 ml uninoculated Ossmer broth). Following enrichment, each tube was inverted 10 times to mix, and the swabs aseptically discarded.

2.3.1.2 Part-processed meats

For each sample, the coarsely-filtered broth rinse was centrifuged 300 × *g* for 5 min to remove large particulates from the supernatant. The supernatant was carefully transferred to a new 50 ml centrifuge tube, and centrifuged 1,600 × *g* for 20 min. The supernatant was discarded and the pellet resuspended in 9 ml Ossmer broth, transferred to a 10 ml centrifuge tube, and enriched over 18 h at 24ºC. Controls were set up as for whole meats.

2.3.2 Multiplex PCR of enrichments

Prior to PCR preparation, 1 ml of enrichment was stored in an eppendorf tube under refrigeration, to be used for plating. The remaining 8 ml of Ossmer enrichment was centrifuged 1,600 × *g* for 20 min, and the supernatant discarded. The pellet was then resuspended in 5 ml sterile double-distilled water, and centrifuged 1,600 × *g* for 20 min. The supernatant was discarded and the pellet resuspended in 100 µl of sterile milli-Q (MQ) water. A 10 µl portion of this suspension was briefly vortexed in 90 µl of sterile MQ water, heated in a waterbath for 12 min at 96ºC, and centrifuged 11,752 × *g* for 12 min (4ºC). The supernatant was used for PCR template.

Each 25 µl multiplex PCR reaction contained 1 × Qiagen taq PCR mastermix (12.5 U Taq DNA Polymerase, 200 µM each of dATP, dCTP, dGTP and dTTP, 1.5 mM $MgCl_2$, buffer), 0.2 µM of each primer (Table 2) and 10 µl of template DNA. Purified DNA from *Y. enterocolitica* NZRM 3569 (pYV-positive) was used as a positive PCR control and MQ water as a negative (sterility) control. The PCR was carried out under the following conditions: 35 cycles of 94ºC 1 min, 55ºC 1 min and 72ºC 1 min, followed by a final extension of 72ºC for 7 min. PCR products were visualised under ultraviolet radiation on a 2% agarose gel with ethidium bromide staining. Products were compared against a 1kb Plus ladder, and the presence or absence of the target PCR products (*ail* and/or *vir*-F) was recorded. Samples producing both target sequences were scored as positive for *Ye*P+.

Table 2: Multiplex PCR primers

Target gene	Function	Primer sequences
ail (chromosome)	Attachment-invasion locus, regulation of an outer-membrane protein associated with virulence	FW: 5'-TGG TTA TGC GCA AAG CCA TGT-3' RV: 5'-TGG AAG TGG GTT GAA TTG CA-3'
VirF (pYV plasmid)	Regulation of surface-secreted (Yops) and outer-membrane (YadA) proteins associated with virulence	FW: 5'-CTT TTG CTT GCC TTT AGC TCG-3' RV: 5'-AGA ATA CGT CGC TCG CTT ATC C-3'

Reference: Harnett *et. al.*, 1996

2.3.3 Culturing method for isolation and identification of *Ye*P+

Each enrichment was assessed for turbidity, and diluted for plating on to cefsulodin-irgasan-novobiocin (CIN) agar accordingly. For example, a highly turbid sample was serially diluted to 10^{-4}, and a non-turbid sample only to 10^{-2}. For each enrichment, 0.5 ml was added to 4.5ml KOH, inverted five times to mix, and immediately plated to CIN agar and diluted further in buffered peptone water. This KOH dilution represented the 10^{-1} dilution, and was necessary to reduce background microflora. A 20µl volume from the appropriate dilutions was spread over CIN agar, and the plates incubated at 24ºC for 48 h. Presumptive *Yersinia* spp. appear on CIN as 2-3 mm diameter dark pink colonies, with a purple spot in the centre, and an opaque, often irregular, ring around the main colony.

Suspect colonies (at least three per enrichment, where possible) were subcultured to tripticase soy agar (TSA), a low-calcium congo red-based agarose (CRBHO), and spotted on to a selective carbohydrate-containing agar (RAM). The CRBHO was incubated at 37ºC and the TSA and RAM at 24ºC, all for 18 h. Typical *Ye*P+ colonies display a low calcium response on CRBHO (pinprick dark orange colonies) and do not metabolise any sugars on RAM (white colonies). All colonies demonstrating these characteristics were selected for isolate confirmation. RAM-positive/CRBHO-negative colonies (presumptively *Y. enterocolitica* pYV-negative) were also selected for confirmation. The TSA cultures were used for further confirmation tests.

2.3.4 Confirmation of isolates

Isolates were tested for urease, citrate metabolism, catalase and oxidase. Isolates positive for urease and catalase, and negative for oxidase and citrate were confirmed as *Ye*P+ by multiplex PCR. To prepare the PCR template, a colony was transferred from the TSA plate into an eppendorf tube, and suspended in 100 µl of milli-Q water. The suspension was heated in a waterbath for 12 min at 96ºC, then centrifuged 11,752 × *g* for 12 min (4ºC). The supernatant was used for PCR template. The PCR reaction, amplification and visualisation conditions were as described in section 2.3.2, except only 1 µl of template was added and milli-Q water used to make up the final volume to 25 µl.

2.4 MPN enumeration of samples for pYV-positive *Y. enterocolitica*

2.4.1 Preparation of enrichments

2.4.1.1 Whole meats

For each sample, the entire upper surface was swabbed with two swabs as described in section 2.3.1.1. However, the swabs were broken off into a 50 ml tube containing 20 ml Ossmer broth and vortexed vigorously for 1 min to release any cells into suspension. After the addition of a further 30 ml Ossmer broth, the swabs were shaken vigorously by hand for 10 seconds and dispensed into a 3×3 MPN system: 10 ml of neat suspension in each of three 10 ml centrifuge tubes (level A), 1 ml into 9 ml Ossmer broth in each of three 10 ml centrifuge tubes (a 1:10 dilution, level B), and 1 ml of a 1:10 dilution into 9 ml Ossmer broth in each of three 10 ml centrifuge tubes (a 1:100 dilution, level C). A positive control consisting of 10 ml Ossmer broth inoculated with 100 µl of an overnight culture (5 ml TSB, 24ºC) of *Y. enterocolitica* was also prepared, along with a negative (sterility) control (10 ml uninoculated Ossmer broth). All tubes were incubated at 24 ºC for 18 h.

2.4.1.2 Part-processed meats

For each sample, the coarsely-filtered broth rinse as described in section 2.2.2 was centrifuged 300 × *g* for 5 min to remove large particulates from the supernatant. The supernatant was dispensed into a 3×3 MPN system as described for the whole meats (section 2.4.1.1).

2.4.2 Detection of *Y. enterocolitica* in MPN tubes

Each tube of the MPN system, for every sample, was plated to CIN agar plates as described for the P/A enrichments. PCR analysis was conducted on all MPN tubes for 16 samples, and only on the level A tubes for the remaining 10 samples. The methods used for PCR and for the screening and confirmation of suspected and presumptive isolates were as described for the P/A enrichments.

2.5 Calculation of MPN

A spreadsheet was developed using Microsoft Excel to calculate MPN values for whole meats and part-processed meats. The calculations are based on information presented by Peeler *et al*. (1992), in particular details on the MPN calculations from Thomas (1942), and on 95% confidence intervals for MPN tests from Cochran (1950). The spreadsheet requires the input of the number of tubes positive for *Ye*P+, and an extra set of values for the wieght of sample tested in each MPN tube where part-processed meats are analysed (for whole meats, a 25 cm^2 surface area is used as default). Figure 1 displays the spreadsheet output for a 5.0 g sample of mince with three tubes positive at level A, two at level B and one at level C. Figure 2 displays the spreadsheet output for a whole meat sample with the same number of positive tubes at each MPN level. For Figure 2, the output value for MPN/ml (i.e. the concentration of bacteria present in the ossmer swab rinse suspension) has been converted to give an MPN value based on the original meat area. This is a valid extrapolation based on the assumption that 1 ml of swab wash is equivalent to a 0.5 cm^2 area of meat.

The total range over which this 9-tube MPN system operates for whole meats is between 0.06 MPN/cm^2 (LCI of 0.01 with one positive tube at level C; though one positive tube at level A is a more likely result and has a value of 0.07 MPN/cm^2) and 8.77 MPN/cm^2 (UCI of 39.74; all tubes positive but one at level C). For part-processed meats, using a default value of 5.0 g, the range is 0.30 MPN/g (LCI of 0.07, or 0.36 with one positive at level A) to 43.84 MPN/g (UCI of 198.70). For whole meat samples, if no tubes are positive the MPN is recorded as <0.06 MPN/cm^2, and if all are positive, the MPN is recorded as >8.77 MPN/cm^2. A similar approach is taken for part-processed meats, but the actual values will depend on the weight of meat analysed.

2.6 Detection limits

Both the P/A and MPN methods are based on enrichments. The use of selective media encourages the growth of *Y. enterocolitica* and helps to suppress other microflora. Therefore the assumption is made that even low numbers of *Y. enterocolitica* will multiply to a level that will be within the limits of detection for CIN plating and the PCR. The inclusion of spiked samples has supported this assumption (see results).

Additionally, output from the USDA pathogen modeling program (Version 7.0, http://ars.usda.gov/Services/docs.htm?docid=6786) *Y. enterocolitica* aerobic model, provides evidence for sufficient enrichment when conditions are set as close as possible to enrichment in Ossmer broth, within the confines of the model. The concentration of *Y. enterocolitica* on samples analysed in the pilot survey is likely to be lower than the minimum initial concentration in the model (3-log). From the model output (Figure 3), the lag phase of 2.3 h and generation time of 0.7 h can be applied to a starting inoculum of 1 cell in 9 ml Ossmer broth (0.11 cells/ml) to predict a final concentration of 5.8 log after 18 h incubation. This is within the limits of detection for the PCR and CIN plating (see below).

Meat pieces (rinsate): Result needs to be MPN/g:

	MPN level:	Level A	Level B	Level C	
INPUT:	g of sample in each level:	1	0.1	0.01	
	No. tubes in each level:	3	3	3	Sum:
INPUT:	No. positive tubes in each level:	3	2	1	6
	Total g of sample in all negative tubes:	0	0.1	0.02	0.12
	Total g of sample in all tubes:	3	0.3	0.03	3.33
	MPN/g =	**9.49**			
	95% confidence intervals:				
	Number of tubes per level:	3			
	+/- quantity:	0.656332			
	log MPN/g:	0.977339			
	Upper:	1.63367			
	Lower:	0.321007			
	Upper CI:	**43.02**			
	Lower CI:	**2.09**			

Figure 1: Example output of MPN spreadsheet calculator for a 5.0 g sample of mince.

Whole meats (swabs): Result needs to be MPN/cm2:

	MPN level:	Level A	Level B	Level C	
	ml of swab wash in each level:	10	1	0.1	
	No. tubes in each level:	3	3	3	Sum:
INPUT:	No. positive tubes in each level:	3	2	1	6
	Total ml of swab wash in all negative tubes:	0	1	0.2	1.2
	Total ml of swab wash in all tubes:	30	3	0.3	33.3
Swab wash:	**MPN/ml =**	**0.95**			
	MPN in 50 ml swab wash:	47.46			
	MPN/cm2:	**1.90** *			
	95% confidence intervals:	**Per ml**	**Per cm2** *		
	Number of tubes per level:	3	3		
	+/- quantity:	0.656332	0.656332		
	log MPN/g:	-0.02266	0.278369		
	Upper:	0.63367	0.9347		
	Lower:	-0.67899	-0.37796		
	Upper CI:	**4.30**	**8.60**		
	Lower CI:	**0.21**	**0.42**		

* The calculations make the assumption that MPN/ml multiplied by the total volume of swab wash (50 ml) equals the total MPN removed from a meat sample, which can then be calculated on a per cm squared basis. The confidence intervals for both are presented.

Figure 2: Example output of MPN spreadsheet calculator for a swabbed sample.

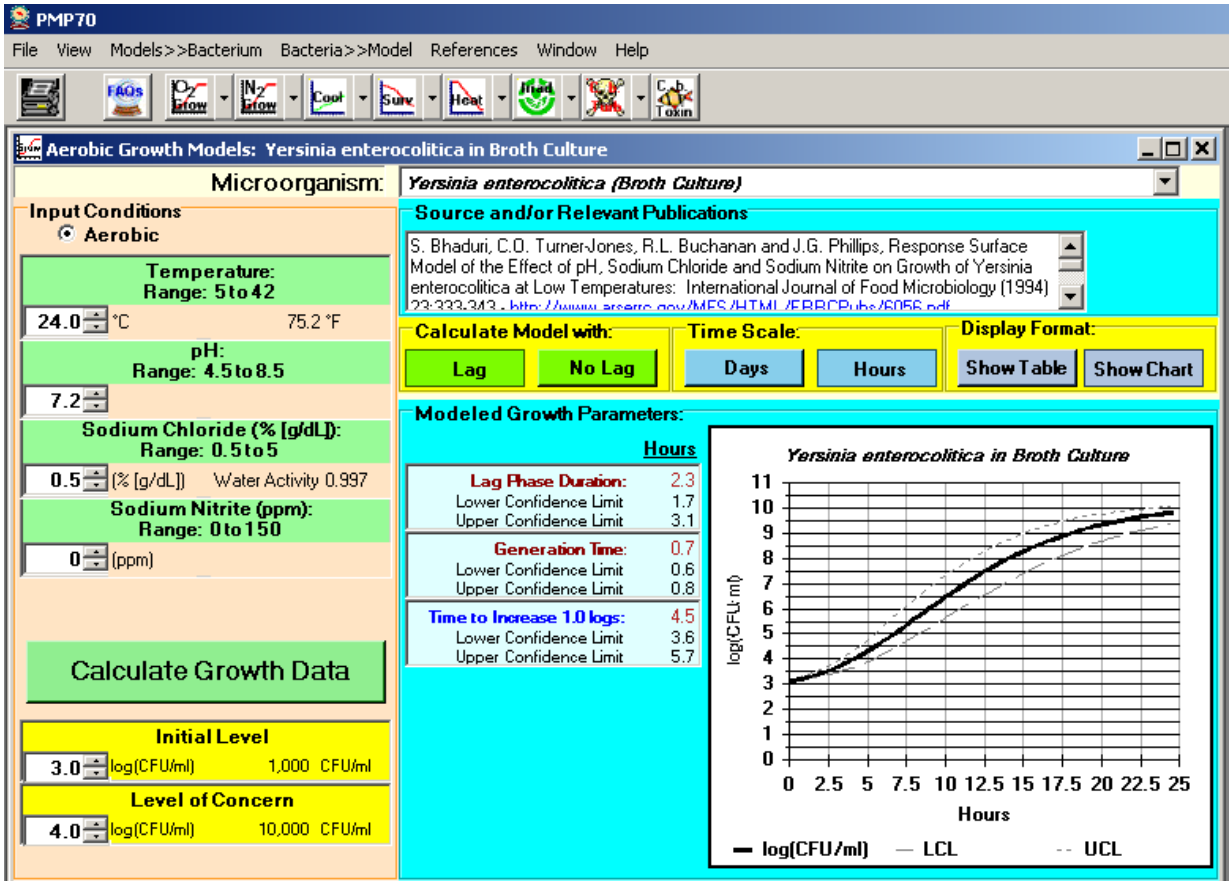

Figure 3: Output from PMP7.0 for aerobic growth of *Y. enterocolitica*.

2.6.1 CIN plates

The detection limit of the CIN plating system is 500 cfu/ml enrichment, i.e. if no presumptive *Yersinia* are observed at the 10^{-1} dilution, then the concentration of *Yersinia* in the enrichment is <500 cfu/ml.

2.6.2 Multiplex PCR

The detection limit of the PCR has been analysed with pYV-positive and pYV-negative *Y. enterocolitica* reference cultures, alone and in combination. Under the conditions of the multiplex PCR, *Y. enterocolitica* at a concentration of between 10^5 and 10^6 cfu/ml of the concentrated enrichment will be detected. This equates to between 10^4 and 10^5 cfu being lysed to produce the DNA template, and a theoretical value between 10^3 and 10^4 of DNA template per 25 µl PCR reaction. The concentrated enrichment is derived from the centrifugation of 8 ml (P/A) or 9 ml (MPN) of Ossmer broth enrichment, resuspended into 100 µl of water (approx. 100-fold concentration). Therefore, the detection limit of the PCR is between 10^3 and 10^4 cfu/ml of enrichment.

3 RESULTS

3.1 Overall results

*Ye*P+ was detected by PCR in 42% of the 41 samples tested. *Ye*P+ was isolated from 22% of the 41 samples tested (Table 3). If a sample that was simultaneously analysed by P/A and MPN was positive by only one of these methods, it was still recorded in Table 3 as *Ye*P+ positive.

Table 3: Number of samples of retail pork positive for *Ye*P+ by PCR and by culturing methods.

Samples	Analysis						***Total (n=41)***	
	P/A (n=10)		MPN (n=11)		Both (n=20)*			
	PCR	Culture	PCR	Culture	PCR	Culture	*PCR*	*Culture*
Whole meats	3	1	4	3	4	2	*11*	*6*
Part-processed meats	2	1	1	0	3	2	*6*	*3*
Total	*5*	*2*	*5*	*3*	*7*	*4*	*17*	*9*
%	*50.0*	*20.0*	*45.5*	*27.3*	*35.0*	*20.0*	*41.5*	*22.0*

* Analysed by both P/A and MPN methods

3.2 Presence/absence results

Table 4 summarises the results for whole meat samples analysed for the presence or absence of *Ye*P+, and Table 5 for part-processed meats.

Table 4: Presence/absence results for whole meats

Week	Sample	Product	Source	Days old*	P/A positive by:		MPN sample No.
					PCR	Culture	
4	P1	Rump steak	Supermarket 1, fridge	2	×	×	S8
4	P2	Rump steak	Supermarket 1, fridge	3	×	×	S9
4	P3	Medallion steak	Supermarket 1, fridge	1	×	×	S10
5	P4	Medallion steak	Supermarket 3, deli.	1	×	×	S11
5	P5	Leg steak	Supermarket 3, fridge	4	×	×	S12
5	P6	Loin chop	Supermarket 3, fridge	2	✓ (w)	×	NA
5	P7	Schnitzel	Supermarket 4, fridge	3	×	×	S13
5	P8	Schnitzel	Supermarket 4, fridge	1	×	×	S14
5	P9	Steak	Supermarket 4, fridge	2	×	×	NA
5	P10	Chop	Supermarket 2, fridge	2	×	×	NA
5	P11	Butterfly steak	Supermarket 2, fridge	2	×	×	NA
5	P12	Schnitzel	Supermarket 2, fridge	1	×	×	NA
6	P13	Schnitzel	Butcher 1	0	×	×	S15
6	P14	Chop	Butcher 1	0	×	×	S16
6	P15	Chop	Butcher 2	0	×	×	S17
6	P16	Butterfly steak	Butcher 3	0	×	×	S18
6	P17	Schnitzel	Butcher 3	0	×	×	S19
7	P18	Schnitzel	Supermarket 1, fridge	3	✓	✓	NA
7	P19	Medallion steak	Supermarket 1, fridge	2	✓	×	NA
7	P20	Schnitzel	Supermarket 2, fridge	3	✓ (w)	✓	S20
7	P21	Butterfly steak	Supermarket 2, fridge	1	✓ (w)	✓	S21
7	P22	Schnitzel	Supermarket 3, fridge	1	✓	×	S22
7	P23	Rump steak	Supermarket 3, deli.	1	×	×	NA
7	P24	Schnitzel	Supermarket 4, fridge	3	×	×	S23
7	P25	Steak	Supermarket 4, fridge	1	×	×	S24
				Total positive:	**6/25**	**3/25**	
				Percentage positive:	**24%**	**12%**	

Table notes: * Number of days between packaging date and date sampled; NA, Not analysed; (w), weak result in PCR.

Table 5: Presence/absence results for part-processed meats

Week	Sample	Product	Source	Days old*	P/A positive by:		MPN sample No.
					PCR	Culture	
8	P26	Mince	Supermarket 1, fridge	1	✓	✓	M3
8	P27	Mince	Supermarket 2, fridge	0	✓	✓	NA
8	P28	Mince	Supermarket 3, fridge	0	✓	×	NA
8	P29	Mince	Supermarket 4, fridge	1	✓	✓	M4
8	P30	Mince	Butcher 1	0	×	×	M5
				Total positive:	**4/5**	**3/5**	
				Percentage positive:	**80%**	**60%**	

Table notes: * Number of days between packaging date and date sampled; NA, Not analysed.

3.3 MPN results

Detection of *Ye*P+ in the MPN tubes for whole meats and part-processed meats by PCR and cultural methods are listed in Tables 6 and 7. Not all MPN enrichments of the B and C levels were analysed by PCR. One sample (S22) was negative for *Ye*P+ by MPN, but positive by P/A analysis. Two samples (S13 and M5) were positive by MPN (weakly PCR-positive only), but negative by P/A analysis. The presence or absence of *Ye*P+ in all other samples analysed by both methods corresponded. The MPN calculations for positive samples are presented in Table 8.

Table 6: Detection of pYV-positive *Y. enterocolitica* in the MPN tubes, whole meats

Week	Sample	Product	Source	Days old*	No. positive tubes at each MPN level (/3) by:						Equivalent P/A sample	
					PCR			Culture			Sample No.	Positive?
					A	B	C	A	B	C		
1	S1	Schnitzel	Supermarket 1, fridge	1	1	NA	NA	1	0	0	NA	
1	S2	Schnitzel	Supermarket 1, fridge	3	0	NA	NA	0	0	0	NA	
1	S3	Loin steak	Supermarket 1, deli.	<1	2 (w)	NA	NA	2	2	0	NA	
2	S4	Schnitzel	Supermarket 2, fridge	1	3	NA	NA	2	0	0	NA	
2	S5	Chop	Supermarket 2, fridge	4	3	NA	NA	0	0	0	NA	
3	S6	Schnitzel	Supermarket 3, fridge	1	0	NA	NA	0	0	0	NA	
3	S7	Steak	Supermarket 3, deli.	1	0	NA	NA	0	0	0	NA	
4	S8	Rump steak	Supermarket 1, fridge	2	0	0	0	0	0	0	P1	×
4	S9	Rump steak	Supermarket 1, fridge	3	0	0	0	0	0	0	P2	×
4	S10	Medallion steak	Supermarket 1, fridge	1	0	0	0	0	0	0	P3	×
5	S11	Medallion steak	Supermarket 3, deli.	1	0	0	0	0	0	0	P4	×
5	S12	Leg steak	Supermarket 3, fridge	4	0	0	0	0	0	0	P5	×
5	S13	Schnitzel	Supermarket 4, fridge	3	3 (w)	3 (w)	1 (w)	0	0	0	P7	×
5	S14	Schnitzel	Supermarket 4, fridge	1	0	0	0	0	0	0	P8	×
6	S15	Schnitzel	Butcher 1	<1	0	NA	NA	0	0	0	P13	×
6	S16	Chop	Butcher 1	<1	0	NA	NA	0	0	0	P14	×
6	S17	Chop	Butcher 2	<1	0	0	0	0	0	0	P15	×
6	S18	Butterfly steak	Butcher 3	1	0	NA	NA	0	0	0	P16	×
6	S19	Schnitzel	Butcher 3	1	0	0	0	0	0	0	P17	×
7	S20	Schnitzel	Supermarket 2, fridge	3	3	2	0	0	1	0	P20	✓
7	S21	Butterfly steak	Supermarket 2, fridge	1	2	1	0	0	1	0	P21	✓
7	S22	Schnitzel	Supermarket 3, fridge	1	0	0	0	0	0	0	P22	✓ (PCR only)
7	S23	Schnitzel	Supermarket 4, fridge	3	0	0	0	0	0	0	P24	×
7	S24	Steak	Supermarket 4, fridge	1	0	0	0	0	0	0	P25	×
8	S25	Schnitzel	Supermarket 2, fridge	<1	0	0	0	0	0	0	NA	
8	S26	Schnitzel	Supermarket 4, fridge	4	0	0	0	0	0	0	NA	
Total positive (at any MPN level):					**7/26**			**5/26**				
Percentage positive (at any MPN level):					**29.9%**			**19.2%**				

Table notes: * Number of days between packaging date and date sampled; NA, Not analysed (see discussion); (w), weak result in PCR.

Table 7: Detection of pYV-positive *Y. enterocolitica* in the MPN tubes, part-processed meats

Week	Sample	Product	Source	Days old*	No. positive tubes at each MPN level (/3) by:						Equivalent P/A sample	
					PCR			Culture			Sample No.	Positive?
					A	B	C	A	B	C		
2	M1	Mince	Supermarket 2, fridge	1	3	NA	NA	0	0	0	NA	
3	M2	Stir-fry pieces	Supermarket 3, deli.	1	0	NA	NA	0	0	0	NA	
8	M3	Mince	Supermarket 1, fridge	1	3	3	3	3	3	3	P26	✓
8	M4	Mince	Supermarket 4, fridge	1	3	0	0	2	0	0	P29	✓
8	M5	Mince	Butcher 1	?	1 (w)	0	0	0	0	0	P30	×
Total positive (at any MPN level):					**4/5**			**2/5**				
Percentage positive (at any MPN level):					**80%**			**40%**				

Table notes: * Number of days between packaging date and date sampled; NA, Not analysed (see discussion); ?, Product may have been frozen and thawed prior to purchase.

Table 8: Calculation of most probable number, all PCR- or culture-positive samples

Week	Sample	Product	Source	Days old*	Most probable number (MPN/cm^2 or MPN/g) by:					
					PCR			Culture		
					MPN	UCI	LCI	MPN	UCI	LCI
1	S1	Schnitzel	Supermarket 1, fridge	1	NC+	-	-	0.07	0.33	0.02
1	S3	Loin steak	Supermarket 1, deli.	<1	NC+	-	-	0.41	1.87	0.09
2	S4	Schnitzel	Supermarket 2, fridge	1	NC+	-	-	0.19	0.86	0.04
2	S5	Chop	Supermarket 2, fridge	4	NC+	-	-	<0.06	-	-
5	S13	Schnitzel	Supermarket 4, fridge	3	5.42	24.59	1.20	<0.06	-	-
7	S20	Schnitzel	Supermarket 2, fridge	3	1.52	6.89	0.34	0.06	0.28	0.01
7	S21	Butterfly steak	Supermarket 2, fridge	1	0.30	1.34	0.07	0.06	0.28	0.01
2	M1	Mince	Supermarket 2, fridge	1	NC+	-	-	<0.27	-	-
8	M3	Mince	Supermarket 1, fridge	1	>42.90	-	-	>42.90	-	-
8	M4	Mince	Supermarket 4, fridge	1	2.48	11.26	0.55	0.82	3.74	0.18
8	M5	Mince	Butcher 1	?	0.31	1.39	0.07	<0.26	-	-

Table notes: * Number of days between packaging date and date sampled; NC+, Not computable (only the A level enrichment tubes were analysed by PCR), but one or more of the A level tubes were positive by PCR.

3.4 Results from spiked samples

3.4.1 Presence/absence spiked samples

Table 9 lists the results from the spiked samples for each week that presence/absence testing was conducted. Even though the samples for spiking were selected from among the samples collected that week, none of the samples used for spiking had any detectable natural *Ye*P+. Therefore any *Ye*P+ detected is considered to be recovery of the spiked inoculum.

Table 9: Recovery of pYV-positive *Y. enterocolitica* in spiked samples by presence/absence

Week	Sample	Product	Source	Days old*	P/A positive by:		Inoculum
					PCR	Culture	
4	P1	Steak	Supermarket 1, fridge	2	✓	✓	14.4 cfu/cm^2
5	P4	Steak	Supermarket 3, deli.	1	✓	✓	10.7 cfu/cm^2
6	P13	Schnitzel	Butcher 1	<1	✓	✓	17.3 cfu/cm^2
6	P13	Schnitzel	Butcher 1	<1	×	✓	1.7 cfu/cm^2
7	P25	Steak	Supermarket 4, fridge	1	✓	✓	14.0 cfu/cm^2
7	P25	Steak	Supermarket 4, fridge	1	✓	✓	1.4 cfu/cm^2
8	P30	Mince	Butcher 1	<1	✓	✓	147.7 cfu/g
8	P30	Mince	Butcher 1	<1	✓	×	15.2 cfu/g
				Total positive:	**7/8**	**7/8**	
				Percentage positive:	**87.5%**	**87.5%**	

* Number of days between packaging date and date sampled.

3.4.2 MPN spiked samples

Table 10 lists the results from the spiked samples for each week that MPN testing was conducted. The samples for spiking were selected from the samples collected that week, and of these, two samples (S4 and M5), had detectable natural *Ye*P+. However, the detectable levels were at a concentration below that of the inoculum, therefore any *Ye*P+ detected in these spiked samples, and all other spiked samples, is considered to be recovery of the spiked inoculum. A comparison between the MPN calculations and the inoculum is presented in Figure 4.

Table 10: Recovery of pYV-positive *Y. enterocolitica* in MPN spiked samples and their MPN values

Week	Sample	Product	Source	Days old*	No. positive tubes at MPN level:						MPN/cm^2 or MPN/g by:						Inoculum
					PCR			Culture			PCR			Culture			
					A	B	C	A	B	C	MPN	UCI	LCI	MPN	UCI	LCI	
1	S1	Schnitzel	Supermarket 1	1	3	NA	NA	0	2	0	NC+	-	-	0.12	0.56	0.03	15.1 cfu/cm^2
2	S4	Schnitzel	Supermarket 2	1	3	NA	NA	3	3	0	NC+	-	-	3.80	17.21	0.84	21.9 cfu/cm^2
3	S6	Schnitzel	Supermarket 3	1	3	NA	NA	3	3	3	NC+	-	-	>8.77	-	-	22.4 cfu/cm^2
4	S8	Steak	Supermarket 1	2	3	3	3	3	3	3	>8.77	-	-	>8.77	-	-	144.0 cfu/cm^2
5	S11	Steak	Supermarket 3	1	3	2	1	3	2	1	1.90	8.60	0.42	1.90	8.60	0.42	10.7 cfu/cm^2
6	S15	Schnitzel	Butcher 1	<1	3	0	0	3	0	0	0.57	2.59	0.13	0.57	2.59	0.13	1.7 cfu/cm^2
7	S24	Steak	Supermarket 4	1	3	1	1	3	1	1	1.17	5.30	0.26	1.17	5.30	0.26	1.4 cfu/cm^2
8	S25	Schnitzel	Supermarket 2	<1	3	0	0	3	0	0	0.57	2.59	0.13	0.57	2.59	0.13	3.0 cfu/cm^2
2	M1	Mince	Supermarket 2	1	3	NA	NA	0	0	0	NC+	-	-	<0.25	-	-	94.1 cfu/g
8	M5a	Mince	Butcher 1	?	3	2	2	1	1	1	10.07	45.66	2.22	0.96	4.36	0.21	131.5 cfu/g
8	M5b	Mince	Butcher 1	?	3	1	0	0	0	0	4.27	19.36	0.94	<0.28	-	-	14.1 cfu/g
Total positive (at any MPN level):					**11/11**			**9/11**									
Percentage positive (at any MPN level):					**100%**			**81.8%**									

Table notes: * Number of days between packaging date and date sampled; NA, Not analysed; ?, Product may have been frozen and thawed prior to purchase; NC+, Not computable (only the A level enrichment tubes were analysed by PCR), but one or more of the A level tubes were positive by PCR.

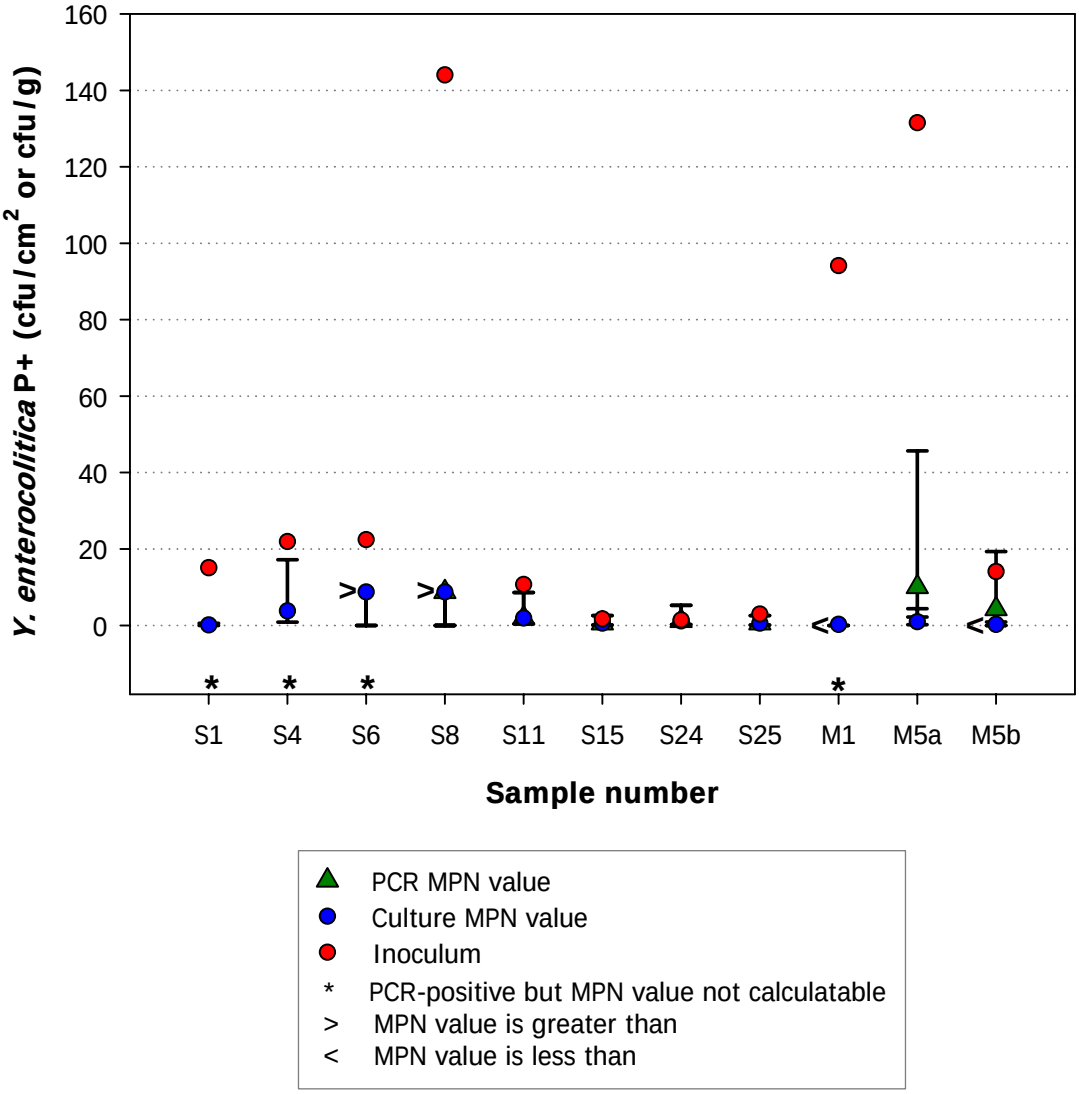

Figure 4: Comparison between the MPN values of the PCR and culture detection with the inoculum for spiked samples tested using the MPN method.

Error bars represent 95% confidence intervals.

4 DISCUSSION

Of the 41 samples of raw retail pork analysed for *Ye*P+ in this pilot survey, 17 (42%) were positive by PCR by either the P/A method or MPN, and *Ye*P+ were isolated from 9 (22%) (Table 3). Six of the 7 (86%) part-processed meat samples were PCR-positive by at least one method. All of these were mince samples; the seventh sample was a stir-fry cut. Eleven out of 34 (32%) whole meat samples analysed were also PCR-positive by at least one method.

The culturing methods are fairly sensitive and can detect >500 cfu/ml *Ye*P+ in an enrichment broth, but it is not always possible to isolate *Ye*P+ colonies from PCR-positive enrichments. The PCR analysis is very specific for *Ye*P+ and uses template from a concentrate of the enrichment. Additionally, the PCR reaction does not appear to be significantly inhibited by the presence of DNA from other microflora or any meat carry-over, such as fats and proteins. The plating method utilises an alkaline treatment and antibiotics to reduce or inhibit non-*Yersinia* microflora, but this does not entirely remove background microflora. Identification of a well-isolated *Yersinia* colony from background microflora on CIN agar plates can often be difficult, particularly when older meat or ground meat are analysed. The genera *Serratia*, *Acinetobacter*, *Citrobacter* and *Enterobacter* have all been isolated on CIN agar plates from pork enrichments.

Thirty samples were analysed by the presence/absence method. Six of the 25 whole meat samples yielded a positive result by PCR (Table 4), as did four of the five mince samples tested (Table 5). By culture, four of the PCR-positive meats did not yield *Ye*P+ colonies. When plated on to CIN agar, these samples produced a number of presumptive *Yersinia* spp., but none were found to be *Y. enterocolitica* or *Ye*P+, so it was not possible to confirm the presence of viable *Ye*P+ in these samples.

Thirty-one samples were analysed by the MPN method. Seven of the 26 whole meat samples were PCR-positive for *Ye*P+ in at least one MPN tube (Table 6), along with four from five part-processed meat samples tested, all mince (Table 7). By culture, four of the PCR-positive meat samples did not yield *Ye*P+ colonies. All CIN agar colonies tested from these culture-negative samples were not *Ye*P+, so the presence of this pathogen in these samples could not be confirmed. Of the 20 samples tested by both P/A and MPN, there were three discrepancies between the MPN and P/A results. These three samples were all negative by culturing for both methods, but PCR-positive by one method only.

Of the 11 samples that were PCR-positive in the MPN testing, MPN values based on PCR could be calculated for only six (Table 8), since previous testing of the MPN method had suggested that the culture methods were more sensitive than the PCR, and so for many samples only the A level MPN tubes were analysed by PCR as an initial screen. Partway through the survey, it became evident that the PCR method was more robust than plating, and it was decided to analyse all MPN tubes by PCR. As a result, only 19 (61%) of the samples analysed by MPN had all MPN tubes analysed by PCR, of which 13 were PCR-negative. For the six PCR-positive samples where an MPN value could be calculated, the concentration of *Ye*P+ in the three whole meat samples was 0.30, 1.52 and 5.42 MPN/cm^2, was 0.31 and 2.48 MPN/g in two of the mince samples, and was >42.90 MPN/g in the third mince sample (i.e. all MPN tubes were positive for this sample).

Where a few comparisons were possible, the MPN values obtained through culturing were usually lower than those calculated from the PCR results. It was not always possible to isolate *Ye*P+ on CIN agar from PCR-positive MPN tubes, and the MPN value is subsequently reduced. Often on these occasions, the PCR result is weak, suggesting that *Ye*P+ had not enriched to a high enough level to be detected on the CIN agar among the background microflora. Alternatively, a positive PCR result might arise through the presence of *Ye*P+ DNA from dead cells. In this regard, the successful

culturing of one *Ye*P+ isolate from any MPN tube for a particular sample provides some evidence to confirm all PCR-positive results for that sample, though false-positive PCR results can not be conclusively eliminated without culturing one *Ye*P+ isolate from *every* PCR-positive tube. We believe the best approach is to use the PCR result for calculation of MPN, provided at least one *Ye*P+ isolate has been cultured from a sample. Where a sample is PCR-positive by MPN, but no *Ye*P+ are isolated, the MPN result is best considered presumptive only.

Naturally-occurring *Ye*P+ were not detected in any of the samples used for spiked controls in the presence/absence testing. Therefore any positives were considered to be recovery of the inoculum. *Ye*P+ was detected in all spiked whole meats over the range 1.4 to 17.3 cfu/cm^2 (Table 9), though one sample was PCR-negative (spiked at 1.7 cfu/cm^2). For the two spiked mince samples tested, an inoculum of 147.7 cfu/g was detected by PCR and recovered on plates, but at 15.2 cfu/g only the PCR analysis was positive; no isolates were recovered on plates. From these results, a conservative detection limit for presence/absence analysis can be summarised as 10 cfu/cm^2 for whole meats, and 100 cfu/g for part-processed meats (mince).

Of the samples used for spiked controls in the MPN analyses, two (S4 and M5) contained naturally-occurring *Ye*P+, however the concentration of natural *Ye*P+ on these samples is believed to be below that spiked (compare Table 8 with Table 10). Therefore, at least some portion of *Ye*P+ detected in these spiked samples is considered to be recovery of inoculum, and any *Ye*P+ detected in other samples is considered to be wholly from the spike. All of the eight spiked whole meats were positive by PCR and isolates were recovered by plating. For five of these samples all MPN tubes were analysed by PCR and the MPN values were identical between PCR and plating. *Ye*P+ was detected in spiked whole meats over the range 1.4 to 144 cfu/cm^2, giving a conservative detection limit of 10 cfu/cm^2. However, the MPN confidence intervals only encompassed the spiked concentration in four samples (range 1.4 to 144 cfu/cm^2), two of which exceeded the computable concentration of this 9-tube MPN system (>8.77 cfu/cm^2). This indicates that recovery of *Ye*P+ is good, but producing an accurate MPN value is difficult (Figure 4). Of the three spiked mince samples, *Ye*P+ was only recovered from one sample, though all three were PCR-positive (range 14.1 to 131.5 cfu/g). This supports the conservative detection limit for part-processed meats (mince) as being 100 cfu/g. Again, there were disparities between the known spike concentration and the calculated MPN.

By combining a robust PCR with a culturing system involving specific *Yersinia*-selective broth and agars, plus isolate screening media, we have produced a method that will successfully detect the presence of *Ye*P+ on whole meat samples with a detection limit which may be as low as 2 cfu/cm^2, though is conservatively stated as 10 cfu/cm^2. Detection of *Ye*P+ in mince is more difficult, hence a conservative detection limit of 100 cfu/g. If necessary, the method used for part-processed meats could easily accommodate non-mince samples such as carcass excisions or other small cuts where a rinse approach is more preferable to swabbing. Importantly, within 24 hours of sample receipt, a presumptive result can be obtained based on PCR. Any suspected *Yersinia* can be isolated within three days of sample receipt, and *Ye*P+ can be confirmed 24 hours later by specific biochemical reactions and PCR. While additional biochemical tests can take a few days more, all are completed within 1 week of sample receipt. Isolates can also be preserved for serotyping if necessary.

The results of this survey indicate that the PCR is slightly more sensitive and reliable as an indicator for the presence of *Ye*P+ than plating methods. For this reason, the PCR results of all MPN tubes should preferentially be used in the MPN enumeration. However, plating must be carried out to ensure viable *Ye*P+ are isolated to confirm that PCR-positive samples do contain these bacteria.

This survey has signalled some problems with the current MPN method. The comparison between spiked inoculum and the MPN values displayed in Figure 4 reveals only three major discrepancies.

Two of these are mince samples, which have either over-estimated or under-estimated the spiked level. In one of these samples (M1) an MPN value could only be calculated from plates, and data from all mince samples indicates that recovery of *Ye*P+ from PCR-positive mince samples is difficult due to background microflora. The major discrepancy (S8) signals a problem with the current 9-tube MPN system. Both of the spiked samples S4 and S8 had similar MPN values, however their actual spiked concentration differed by 1-log. The 9-tube MPN system used in this survey has an upper limit of 8.77 MPN/cm^2 for whole meats and around 43 MPN/g for part-processed meats. These are both very low limits, and may be exceeded regularly if the system is used for potentially highly contaminated samples such as mince or those collected during outbreak investigations.

To increase the upper limit of an MPN system, there are four options. The first is to decrease the area or weight of sample analysed. For example, if only 1 g of mince was analysed, rather than 5 g, the upper limit increases from 43.84 MPN/g to 219.20 MPN/g. The lower limit will increase to 1.50 MPN/g (from 0.30 MPN/g). For mince samples, this might be possible, but the chance of non-detection is increased. Certainly, decreasing the area swabbed for whole meats to below 25 cm^2 will reduce the chance of detecting the pathogen since these samples are more likely to carry low numbers of *Ye*P+. A similar outcome would be achieved by increasing the dilution of the sample/swab broth rinse in the MPN tubes, for example, starting with a 1:100 dilution for level A (currently 1:10 for mince). The risk of non-detection will also increase with this approach. Thirdly, the number of levels might be increased. For example, adding another level of three tubes (a 3×4 MPN) will increase the detection limit for a 5 g mince sample to 190.54 MPN/g and the whole meat limit to 38.11 MPN/cm^2. A fourth option is to increase the number of tubes at each level. This generates a less dramatic change; increasing the number of tubes to five per level (a 5×3 MPN) will take the upper limit to 59.43 MPN/g for part-processed meats (from 43.84 MPN/g), and to 11.89 MPN/cm^2 for whole meats (from 8.77 MPN/cm^2). Increasing the number of tubes at each level is more important for increasing precision. All of these options will increase the work intensity and cost for both PCR and plating.

A solution to the issue indicated above is to adapt the MPN system to the samples for analysis and the objectives of the survey. For example, if the samples are likely to contain low numbers of *Ye*P+, then the current MPN system is adequate, or a simple presence/absence might be all that is needed. Additionally, the area swabbed on such samples can easily be increased and would probably not influence the outcome, other than providing a better indication of contamination. Alternatively, if the objective is to measure both frequency and concentration of *Ye*P+, but quantification is only required where the level of *Ye*P+ is of concern (e.g. 10^2 cfu/g or cfu/cm^2), then samples could be analysed for presence/absence, but greater dilutions used for the MPN to ensure the detection limit is satisfactorily high. If there is good evidence that contamination of samples is likely to be high (e.g. 10^3 cfu/g or cfu/cm^2), then direct plating without enrichment is a viable option. This has been trialled in our laboratory, but with varied success.

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