JML Ling 凌美麟

Rapid detection of food-borne pathogens in clinical specimens, food and environmental samples

Key Messages

1. PCR and RT-PCR methods for 5-hour and 3-hour detection, respectively, of salmonellae and *Vibrio cholerae* in stool, food and environmental water samples have been developed.
2. Such methods can be used in routine laboratories for rapid detection of salmonellae and *V cholerae* and are essential for infection control purposes.

Introduction

Salmonella species are the most common bacterial cause of diarrhoea and *Vibrio cholerae* occasionally cause diarrhoeal outbreaks in Hong Kong. Salmonellae can also cause invasive diseases such as septicaemia or meningitis especially in young children. Gastroenteritis caused by both types of organisms is transmitted via contaminated foods and infected persons.

Laboratory diagnosis of salmonellosis and cholera and location of the infectious source in foods depend on traditional methods of culture and identification. These methods take at least 2 days to yield results thus delaying the institution of prompt prevention and control measures.

For these reasons we aimed to develop rapid molecular biological methods for the detection of salmonellae and *V cholerae* in clinical, food and water samples. We also aimed to optimise these methods for routine use in a diagnostic and public health laboratory and to use these methods for detecting organisms prospectively in various clinical, food and environmental samples taken for surveillance purposes.

Methods

All surviving salmonellae isolated during 2000-2003 were tested for the presence of genes specific to *Salmonella* species by a multiplex polymerase chain reaction (PCR).[1] The detection limit of the PCR was determined using 10-fold serial dilutions of overnight colonies of a standard strain of *S choleraesuis* (ATCC 13076). The specificity was determined by testing a variety of bacterial organisms other than salmonellae. All stool specimens (including rectal swabs) received for bacterial culture by our laboratory during the period May 2005 to August 2006, a variety of food items, and water samples collected from three different beaches were cultured for stool pathogens using standard procedures and subjected to PCR for detection of salmonellae. Real-time PCR (RT-PCR)[2] was also used to detect salmonellae in pure culture, stool samples, foods and water.

All surviving *V cholerae* in our culture collection (since 1983) were subjected to multiplex and hexaplex PCR.[3,4] The detection limit and specificity of the PCR were tested as described above. Since no *V cholerae* were cultured from stool specimens during the period of study, nor from the same food items and water samples used above, 0.5 μL of DNA extracted from each of the serial 10-fold dilutions of *V cholerae* was added before performing the PCR. Also RT-PCR[5] was used to detect vibrios in pure culture, stool samples, foods and water.

Results

All 410 isolates of 58 serotypes of salmonellae isolated during 2000-2003 were positive on PCR for the *invA* gene while only 15% were positive for the *spvC* gene (Table 1). *S enteritidis*, *S typhimurium* and *S derby* were the most common salmonellae tested, each with 10% or more isolates. More than 90% of *S enteritidis*, only 22% of *S typhimurium* but none of the *S derby* isolates had both the *spvC* and *invA* genes.

Hong Kong Med J 2009;15(Suppl 2):S26-9

Department of Microbiology, The Chinese University of Hong Kong
JML Ling

RFCID project number: 01030942

Principal applicant and corresponding author:
Dr Julia Mei-lun Ling
Department of Microbiology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, NT, Hong Kong SAR, China
Tel: (852) 2632 2351 / 2632 3333
Fax: (852) 2647 3227
E-mail: meilunling@cuhk.edu.hk

Table 1. *Salmonella* serotypes tested for presence of *spvC* and *invA* genes

Salmonella serotype	Total tested		*spvC- invA+*		*spvC+ invA+*	
	No.	%	No.	%	No.	%
S enteritidis	52	13	4	8	48	92
S typhimurium	46	11	36	78	10	22
S derby	40	10	40	100	0	0
S stanley	35	9	35	100	0	0
S rissen	28	7	28	100	0	0
S saintpaul	22	5	22	100	0	0
S virchow	22	5	22	100	0	0
S typhi	12	3	12	100	0	0
S heidelberg	11	3	10	91	1	9
S hadar	10	2	10	100	0	0
S weltevreden	10	2	10	100	0	0
S anatum	8	2	8	100	0	0
S give	8	2	8	100	0	0
S agona	7	2	7	100	0	0
S infantis	7	2	7	100	0	0
S paratyphi A	7	2	7	100	0	0
S newport	6	1	6	100	0	0
S reading	5	1	5	100	0	0
S thompson	5	1	5	100	0	0
S bareily	4	1	4	100	0	0
S braenderup	4	1	4	100	0	0
S london	4	1	4	100	0	0
S bovis-morbificans	2	0	1	50	1	50
Others*	52	13	52	100	0	0
Total	410	100	350	85	60	15

* Includes three strains each of *S bardo*, *S haardt*, *S manhattan*, *S nchanga*, *S niensteden* and *S panama*, two strains each of *S blockley*, *S galiena*, *S indiana*, *S krefeld*, *S lomita*, *S mbandaka*, *S tenessee* and *S wandsworth*, and one strain each of *S aberdeen*, *S bonn*, *S eppendorf*, *S giza*, *S hartford*, *S kentucky*, *S lagos*, *S lockleaze*, *S montevideo*, *S muenchen*, *S muenster*, *S oslo*, *S potsdam*, *S seegefeld*, *S shubra*, *S singapore*, *S sinstorf*, *S texas*, *S uganda*, *S uppsala* and *S zanzibar*, all of which had the *invA* gene only

The detection limit for *invA* using PCR was $9.6x10^2$ cfu/mL while that for *spvC* was $9.6x10^3$ cfu/mL. One PCR cost approximately HK$6 while culture and identification of one salmonella strain from one stool specimen cost approximately HK$38.

The following 32 organisms were tested for *spvC* and *invA* and none was positive: *Aeromonas hydrophila*, *Citrobacter* sp, *C freundii*, *C koseri*, *Campylobacter jejuni*, *Enterobacter* sp, *E aerogenes*, *E cloacae*, *Escherichia coli*, *Helicobacter pylori*, *Klebsiella* sp, *K oxytoca*, *K pneumoniae*, *Plesiomonas shigelloides*, *Pseudomonas aeruginosa*, *P putida*, *Proteus mirabilis*, *P vulgaris*, *Providencia stuartii*, *Shigella boydii*, *S dysenteriae*, *S flexneri*, *S sonnei*, *Serratia sp*, *S marcescens*, *Vibrio cholerae*, *V parahaemolyticus*, *Bacillus cereus*, *Enterococcus sp, E faecalis*, *E faecium* and *Staphylococcus aureus*.

More than 11 000 stool specimens were received for bacterial culture from May 2005 to August 2006. The stool culture positive rate for salmonella ranged from 0.9% to 4.9%, averaging 3.1%. Five different sample preparation and PCR conditions were tried and optimised on >3000 stool specimens. The method showing highest sensitivity was used on the remaining specimens, giving an overall sensitivity of 81%. The detection limit was $4.8x10^4$ cfu/mL and $9.6x10^4$ cfu/mL for *invA* and *spvC*, respectively. Specimens that were negative for salmonellae by PCR but positive by culture had very low cell counts and were loose or soft stools. The PCR on stools took approximately 5 hours to produce a result.

All food and water samples (Table 2) were negative for salmonellae. DNA from different cfu/mL of salmonellae was seeded into these samples to test the detection limit of the PCR method. It ranged from $9.6x10^3$ cfu/mL to $9.6x10^6$ cfu/mL.

RT-PCR was performed on pure cultures of 43 of the 58 salmonella serotypes (Table 1) and four each of serogroups B, C, D and E (serotypes could not be determined) and all gave a positive reaction. It was also performed on 94 salmonella culture-positive stool samples (50 PCR positive,

Table 2. Detection limit for *invA* and *spvC* in food items and environmental water samples

Source of sample	Detection limit ($x10^3$ cfu/mL) for*									
	invA					*spvC*				
	9600	960	96	48	9.6	9600	960	96	48	9.6
Food										
Raw oyster (Irish)	+	-	-	-	-	+	-	-	-	-
Ice-cream	+	+	-	-	-	+	+	-	-	-
Fresh pork, intestine (chicken), raw oyster (Australian)	+	+	+	-	-	+	+	-	-	-
Chocolate milk, juice	+	+	+	+	-	+	+	-	-	-
Small intestine (pig)	+	+	+	+	+	+	+	-	-	-
Sweet bean curd, roasted duck, ham, salmon sashimi	NT	NT	+	+	-	NT	NT	+	-	-
Drinking yoghurt, colon and kidney of pig, barbecued pork, bivalve, salami, cooked simesaba sashimi	NT	NT	+	+	-	NT	NT	+	+	-
Roasted pork	NT	NT	+	+	+	NT	NT	+	+	+
Water										
Stanley Main Beach	NT	NT	+	+	+	NT	NT	+	+	+
Turtle Cove	NT	NT	+	+	-	NT	NT	+	+	-
Wong Shek	NT	NT	+	+	+	NT	NT	+	+	+

* + denotes detected, - not detected, and NT not tested

Table 3. Detection limit for *rfb* and *ctxA* genes by polymerase chain reaction of food and environmental water samples

Source of sample	Detection limit (x10³ cfu/mL) for*							
	O1				O139			
	80		8		120		12	
	rfb	*ctxA*	*rfb*	*ctxA*	*rfb*	*ctxA*	*Rfb*	*ctxA*
Food								
Sweet bean curd, drinking yoghurt, chocolate milk, juice, fresh pork, intestine (chicken), colon and kidney of pig, roasted pork, barbecued pork, roasted duck, ham, salami, cooked simesaba sashimi	+	+	+	+	+	+	+	+
Bivalve	+	+	+	-	+	+	+	+
Ice-cream	+	+	+	-	+	+	-	-
Salmon sashimi	+	+	-	-	+	+	+	+
Raw oyster (Irish and Australian)	+	+	-	-	+	+	-	-
Water								
Stanley Main Beach	+	+	+	+	+	+	+	+
Turtle Cove	+	+	+	+	+	+	+	+
Wong Shek	+	+	+	+	+	+	+	+

* + denotes detected, and - not detected

44 PCR negative). All 50 PCR positive and 33 PCR negative samples were RT-PCR positive giving a total positive rate of 88% in contrast to 53% on PCR. The detection limit was $4.0x10^2$ cfu/mL. Nevertheless, 11 (12%) were both PCR and RT-PCR negative. The RT-PCR for one stool specimen took approximately 3 hours and cost approximately HK$8.

The RT-PCR on all food and water samples tested had a detection limit of $9.6x10^2$ to $9.6x10^3$ cfu/mL.

A total of 24 isolates of *V cholerae* were subjected to multiplex and hexaplex PCR and all gave positive results. The detection limit in pure culture was 80 to $1.2x10^2$ cfu/mL but was $8x10^3$ to $1.2x10^4$ cfu/mL for stool samples. Nevertheless, since only multiplex PCR could differentiate between O1 and O139, we propose that multiplex PCR be used to detect *V cholerae*.

40 organisms were tested using PCR and all gave negative results: *V alginolyticus*, *V campbelli*, *V fluvalis*, *V furnissii*, *V harveyi*, *V marinus*, *V parahaemolyticus*, *V pelagius*, *V splendidus*, *A hydrophila*, *Citrobacter* sp, *C freundii*, *C koseri*, *Enterobacter* sp, *E aerogenes*, *E cloacae*, *E coli*, *H pylori*, *Klebsiella* sp, *K pneumoniae*, *K oxytoca*, *P aeruginosa*, *P putida*, *P mirabilis*, *P vulgaris*, *P stuartii*, *P shigelloides*, *S boydii*, *S dysenteriae*, *S flexneri*, *S sonnei*, *S typhimurium, Serratia* sp, *S marcescens*, *B cereus*, *Corynebacterium jeikeium*, *Enterococcus* sp, *E faecalis*, *E faecium* and *S aureus*.

Approximately 200 stool samples were seeded with *V cholerae* O1 or O139 DNA then subjected to PCR; 99% were positive for O1 *V cholerae* and 85% for O139. The detection limit was $8x10^3$ cfu/mL for O1 and $1.2x10^4$ cfu/mL for O139. The food and water samples lacking *V cholerae*, were also seeded with *V cholerae* O1 or O139 DNA and subjected to PCR. The detection limit of O1 varied from $8x10^3$ to $8x10^4$ cfu/mL and that of O139 from $1.2x10^4$ to $1.2x10^5$ cfu/mL (Table 3).

The 24 *V cholerae* strains tested by PCR were also tested by RT-PCR. All were positive. All three PCR-positive stool samples and 30 of 33 PCR-negative samples were RT-PCR positive when $8x10^2$ cfu/mL were present. The remaining three were RT-PCR positive when $8x10^3$ cfu/mL were present. The RT-PCR performed on all food and water samples had a detection limit of $8x10^2$ to $8x10^3$ cfu/mL.

Discussion

None of the 32 non-salmonella bacterial species tested were positive for the *invA* gene (present on the chromosome), but all the salmonella serotypes were, indicating that detection of this gene is a sensitive and specific method for salmonellae. Only a small percentage of salmonellae (15%) were positive for the *spvC* gene, however, *spvC* is present on a virulence plasmid which may not be present in all strains.

Our PCR method could detect salmonellae at levels as low as $9.6x10^2$ cfu/mL in pure culture, although at least $4.8x10^4$ cfu/mL was required for its detection in stools. As PCR inhibitors are often present in stools, the detection limit is expected to be higher.

Although our PCR method achieved only 81% sensitivity, it yields same-day results. We did not incubate our specimens to increase the number of organisms, thus those containing few organisms gave negative results. Other studies with higher sensitivity rates achieve these by incubating specimens but results are not available until the next day. Specimens containing large numbers of organisms are highly infectious compared with those containing smaller numbers so a same-day result is desirable. To avoid missing salmonella-positive stools that are PCR-negative due to inhibitors or other unknown factors, we propose that the same specimen be subjected to both PCR and culture. Polymerase chain reaction provides a rapid result while the culture may confirm any false-negatives.

The PCR detection limit for salmonellae in food and environmental water was comparable with or higher than that in stool samples. It is difficult to explain why detectable salmonella numbers had to be higher in fresh foods than processed foods, as additives contained in the latter should inhibit the amplification reaction. Since such high salmonella counts are unlikely to be present in food or water samples, we propose that a culture and PCR be performed on the same sample.

The RT-PCR method gave a higher positive rate than the PCR method, indicating that the former is a more sensitive method. Both methods give same-day results and performing RT-PCR is only slightly more expensive than a PCR. Both are much cheaper than culture and identification.

Since the detection limit for *V cholerae* in stools was 10- to 100-fold lower than that for salmonellae, our method is sufficiently sensitive for detecting *V cholerae*. Patients with cholera usually excrete the organism in numbers well above the detection limit. Although both the hexaplex PCR and multiplex PCR can detect *V cholerae*, only the multiplex PCR can differentiate O1 from O139. Therefore the multiplex PCR, which gave positive rates of 99% and 85% for O1 and O139 respectively, should be used to detect *V cholerae*.

Although we had no stool specimens positive for *V cholerae*, our results with virtual *V cholerae*–containing stool samples indicated that our method was very sensitive. As with salmonellae, we propose that concomitant cultures and multiplex PCR be performed on specimens to avoid false-negative PCR results. Polymerase chain reaction was very specific for detecting *V cholerae* as none of 40 other organisms tested (mostly normal bowel commensals) were positive.

The *V cholerae* detection limit in food and water samples was similar to or higher than that in stools, indicating the presence of other substances that may affect the amplification procedure.

Using RT-PCR, we could detect *V cholerae* at a lower limit than when using PCR. Since it is not much more expensive to perform, it is better to use RT-PCR for detecting both *V cholerae* and salmonellae in stool or food samples. Nevertheless, RT-PCR requires special, costly, equipment that may be too expensive for some diagnostic laboratories while PCR requires only a simple thermal cycler that is currently reasonably inexpensive. Hence PCR may be better for diagnostic laboratories switching to molecular detection of stool pathogens.

We have developed specific methods using PCR and RT-PCR for same-day detection of salmonellae and *V cholerae* in stool, food and water samples. These methods gave ≥80% detection rates with the remaining ≤20% being undetected due to their very low organism counts.

Acknowledgements

This study was supported by the Research Fund for the Control of Infectious Diseases (RFCID: 01030942), Food and Health Bureau, Hong Kong SAR Government. We also thank NWS Lo who provided us with professional advice and voluntarily assisted us technically amid his tight working schedule.

References

1. Chiu CH, Ou JT. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR combination assay. J Clin Microbiol 1996;34:2619-22.
2. Cheung PY, Chan CW, Wong W, Cheung TL, Kam KM. Evaluation of two real-time polymerase chain reaction pathogen detection kits for *Salmonella* spp. in food. Lett Appl Microbiol 2004;39:509-15.
3. Hoshino K, Yamasaki S, Mukhopadhyay AK, et al. Development and evaluation of a multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. FEMS Immunol Med Microbiol 1998;20:201-7.
4. Singh DV, Isac SR, Colwell RR. Development of a hexaplex PCR assay for rapid detection of virulence and regulatory genes in *Vibrio cholerae* and *Vibrio mimicus*. J Clin Microbiol 2002;40:4321-4.
5. Lyon WJ. TaqMan PCR for detection of *Vibrio cholerae* O1, O139, non-O1, and non-O139 in pure cultures, raw oysters, and synthetic seawater. Appl Environ Microbiol 2001;67:4685-93.