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Detection of *Salmonella invA* by isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) in Zambia

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Abstract

The isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) is a new isothermal DNA amplification method composed of exo Bca DNA polymerase, RNaseH and DNA–RNA chimeric primers. We detected *invA* of *Salmonella* from chicken carcasses, egg yolk and cattle fecal samples. Fifty-three of 59 isolates were *invA*-positive in ICAN-chromatostrip detection. The result was consistent with those obtained by standard PCR. *Salmonella invA* was detected in 12 of 14 carcass rinses by ICAN, while in 7 of 14 rinses by standard PCR. These results indicate that ICAN is an efficient, sensitive and simple system to detect *invA* of *Salmonella* species in developing countries such as Zambia.

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Résumé

L'amplification des acides nucléiques par amorce chimérique (ICAN) est une nouvelle méthode d'amplification isothermique de DNA composée de exo Bca DNA polymérase, RNaseH et d'amorces chimériques DNA–RNA.

Nous avons détecté le gène *invA* de *Salmonella* de à partir de poulets, de jaunes d'œufs et d'échantillons de matière fécale de bovin. 53 des 59 isolats furent *invA* positifs en détection ICAN, les résultats furent concordant avec ceux obtenus par la PCR standard. Le gène *invA* de *Salmonella* a été détecté dans 12 des 14 carcasses examinées ICAN tandis que 7 sur 14 étaient positifs lors de l'examen par PCR standard. Ces résultats indiquent que la méthode ICAN est une méthode efficace et sensible et constitue un systéme simple pour détecter I'*invA* des *Salmonella* dans les pays en développement tels que la Zambie.

Mots-clés: *Salmonella*; *invA*; ICAN; PCR; Zambie

1. Introduction

Salmonella is one of the major food borne causes of gastroenteritis and is frequently associated with contaminated poultry meat [1]. Contamination of poultry or poultry meat may occur throughout the whole production chain and important risk factors for contamination at each stage of this process have been identified [1–3].

Contamination of chicken and cattle by *Salmonella* is a significant source of human diarrhea in Zambia [4–6]. *Salmonella enterica* serotype Enteritidis has been isolated from table eggs (3.8%) and chicken carcasses (4.7%) [4]. Chickens are usually asymptomatic carriers of *Salmonella*. Outbreaks of salmonellosis have been reported in Zambia and most of them were in chickens [4,6]. Bovine salmonellosis is also economically important disease in Zambia [7]. Therefore, studies on rapid methods for detection of *Salmonella* in animal derived foods and ready-to-eat-foods are of value to decide whether the appropriated products are fit for human consumption. As the *Salmonella* status of carcasses from slaughter animals is of great importance for the quality of the processed products rapid methods for detecting *Salmonella* organisms are needed. In Zambia, this is the first trial of *invA* detection from isolates and from carcass contaminated with *Salmonella*.

Application of a two-tiered detection system based on a combination of serologic test and bacteriologic confirmation have been essential for the success of the *Salmonella* control program. Conventional cultural methods for detecting *Salmonella* involve enrichment in selective broth, followed by isolation on selective differential agar. Both a primary and a secondary culture are necessary for isolating *S. enterica* from foods. Isolation and identification can therefore be labor-intensive and expensive. Hence, there is a need for a sensitive non-cultural detection for the pathogen in Zambia.

It has been reported that PCR for the specific detection of *Salmonella* is evaluated targeting an *invA* gene [8]. ICAN targeting *invA* has developed an innovative method for

DNA amplification that is performed at a constant temperature. ICAN may offer an alternative method to PCR that requires cycles of different temperature to amplify DNA. Using ICAN method, we can amplify DNA present in extremely small amount and its efficiency is equivalent to or more than that of PCR. In this study, we tried ICAN to detect *invA* from *Salomonella* isolates and to screen *invA* from rinse solution of chicken carcasses in Zambia.

2. Materials and methods

2.1. Salmonella isolates

The *Salmonella* 59 strains isolated in Lusaka from 1994 to 2004 were used. After cloning and identification, Salmonella isolates in BHI medium were stored at −80 °C. A total of 240 egg yolk samples (mixture 10 eggs egg yolk, 2400 eggs), 382 chicken carcasses, six dead chicken (sudden death with diarrhea), 25 cattle feces from various slaughter houses were examined and the 59 strains were obtained. A part of them, we reported phage typing [4]. Briefly, phage typing of these strains revealed predominantly enteropathogenic phage type 4 (PT4) from egg yolk and chicken meat. Nine strains were originated from table egg yolk and seven strains were from carcasses. In the strains from chicken carcasses, three were phage type 7 (PT7) and eight were rough type (RT). The serotypes were Enteritidis (27 strains) and Mubadaka (three strains).

2.2. DNA extraction

After identification, *Salmonella* isolates were cultured in BHI agar for 24 h at 37 °. Small amount of bacteria (10^2–10^4) was inoculated into distilled water and heated at 95 °C 10 min. After centrifugation at 15,000 rpm for 5 min, supernatant was used as DNA sample.

2.3. ICAN and chromatostrip detection

ICAN was performed by using TaKaRa ICAN (*Salmonella* Detection Kit-chromatostrip version, Takara Bio Co., Ltd, Kyoto, Japan). Briefly, *invA* gene in sample (5 μl) was amplified in reaction-mixture (20 μl) containing enzyme Mix and primer Mix at 58 °C for 60 min in a water bath. The ICAN-product was used as a source of amplified *invA*. In the detection, the ICAN-products (5 μl) and denature diluent (20 μl) were mixed and incubated for 1 min in 96 wells micro-titer plate. Avidin fixed chromatostrip was put into each well. Subsequently, the strips were reacted with detection probe or internal probe for 10 min (FITC-labeled probe hybridization reaction). Final reaction was done by using gold-conjugated labeled anti-FITC antibody for 10 min at room temperature. ICAN was highly specific to *Salmonella* strains (three positive controls) without any cross-reaction to *Escherichia coli* (10 strains) and *Proteus mirabilis* (one strain).

2.4. PCR

Modified PCR based on the method of Rahn et al. [8], was used to identify isolates. Briefly, the *invA* gene was amplified by PCR with primers 139: GTG AAA TTA TCG CCA CGT TCG GGC AAA and 141:TCA TCG CAC CGT CAA AGG AAC C. The specimens (2 μl) were diluted with 8 μl of PCR buffer containing 10 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin. The diluted specimens were heated at 94 °C for 10 min and then kept at 80 °C for another 30 min. The PCR mixture was directly added to heat-treated specimens, so that they contained 0.25 mM each deoxynucleoside triphosphate, 1 mM each primer, and 0.5 U of Taq DNA polymerase in a total volume of 20 μl. The PCR amplification condition was 94 °C (1 min), 55 °C (1 min), and 72 °C (1 min) for 35 cycles. The amplified DNA fragment was analyzed by 1% agarose gel electrophoresis. With this system, a DNA fragment of 284 bp was amplified in samples containing strains of *Salmonella* species. The corresponding DNA fragment was not amplified in samples containing the other bacterium such as *E. coli.*

2.5. Sampling of carcasses from various markets and DNA extraction

The chicken carcasses were sampled from various markets in Lusaka, Zambia. Prepacked carcasses were divided from the largest processing plant in Lusaka. A carcass (25 g) was sampled aseptically and rinsed in 50 ml PBS. The rinse solution was used for the origin of culture and ICAN. The rinse solution (0.1 or 0.2 ml) was cultured directly on SS agar for 24 h at 37 °C. The identity of suspected *Salmonella* isolates was confirmed biochemically and serologically by standard method. For primary culture, 1 ml rinse solution was inoculated into BHI liquid medium for 24 h at 37 °C and subsequently inoculated on SS agar. The secondary culture was inoculated on SS agar for 24 h at 37 °C.

For DNA extraction from rinse solution, 40 ml was centrifuged at 5000 rpm/20 min, precipitate was resuspended in 1 ml of distilled water, and heated at 95 °C for 10 min. After centrifugation at 15,000 rpm for 10 min, supernatant was used as extracted DNA.

3. Results

We examined 59 isolates from chicken carcass, egg yolk, dead chicken, and cattle feces in Zambia. Table 1 shows *invA*-positive rate in ICAN-chormatostrip detection

Table 1
Number of *invA* positive *Salmonella* in ICAN and standard PCR

Isolates from	Total number examined	*invA*-positive	
		ICAN	Standard PCR
Healthy chicken carcass	21	18/21 (85.7)	18/21 (85.7)
Egg yolk	9	6/9 (66.7)	6/9 (66.7)
Dead chicken carcass	4	4/4 (100.0)	4/4 (100.0)
Cattle feces	25	25/25 (100.0)	25/25 (100.0)

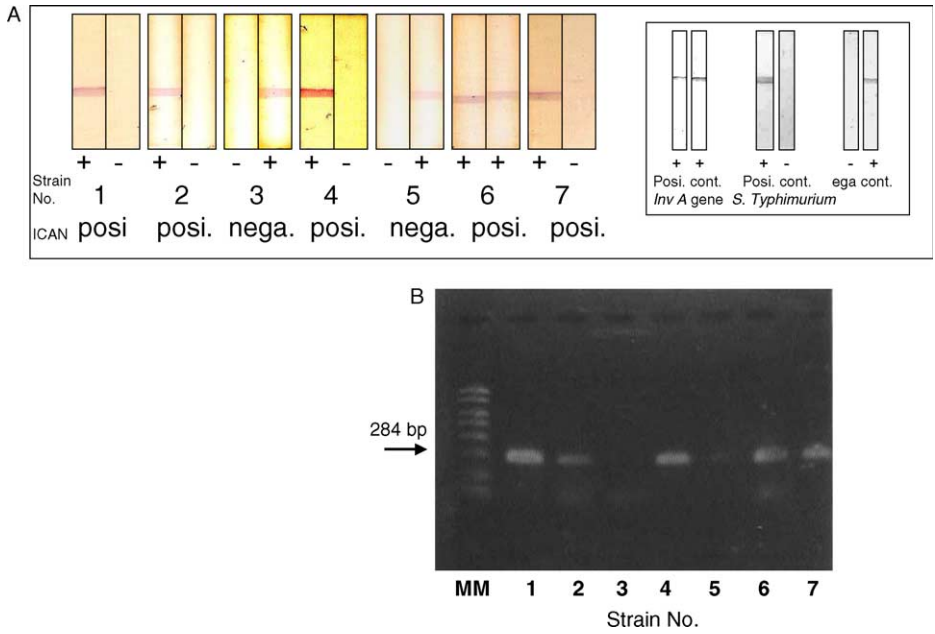

Fig. 1. Typical patterns of *invA* positive and negative strains in ICAN and standard PCR. In ICAN-choromatostrip detection (A), left strip means detection strip and right one means internal control strip. Two reference positive patterns are obtained as double positive (purified *invA* gene) or single positive (only detection strip positive, a *Salmonella* strain: *Salmonella* Typhimurium). Negative pattern is single positive band in right strip (internal control). Standard PCR (B) showed 284 bp band after gel electrophoresis. Positive lanes are 1, 3, 4, 6, and 7 (lane number means strain number).

and standard PCR. Fifty-three of 59 isolates were *invA*-positive in both methods. Remaining samples (six isolates) showed negative results in ICAN (Fig. 1A) and standard PCR (Fig. 1B), including isolates from chicken carcass or egg yolk (Table 1).

It is well known that *invA* is a maker gene of *Salomonella* and almost of all showed *invA* positive [9,10]. The *invA*-positive rate in our isolates from chicken carcass and egg yolk was 85.7 and 66.7%, respectively. Therefore, we hope to determine relationship to phage typing. As shown in Table 2, phage type RT and RT4 showed 75% *invA*-positive, respectively. RT7 and unknown phage type showed 100% *invA*-positive.

Salmonella invA was detected in 12/14 rinse solution from chicken carcass by ICAN and in 7/14 rinse solution from the samples by standard PCR (Table 3). In samples from

Table 2
The relationship to *invA* and phage typing

Phage typing	*invA*-positive rate (%)
RT	6/8 (75)
RT4	12/16 (75)
RT7	3/3 (100)
Unknown	3/3 (100)

Table 3
Detection of *Salmonella* from carcass rinse

Sample from various markets	Detection of *invA*		Number of *salmonella* (CFU/ml) in carcass rinse	
	By ICAN	By standard PCR	Primary culture	Secondary culture
A	+	+	Numerous	Numerous
B	+	+	Numerous	Numerous
C	+	+	Numerous	Numerous
D	+	+	Numerous	Numerous
E	+	+	550	Numerous
F	+	+	200	Numerous
G	+	+	950	Numerous
H	+	−	50	Numerous
I	+	−	0 (<50)	Numerous
J	+	−	0 (<50)	Numerous
K	+	−	0 (<50)	Numerous
L	+	−	0 (<50)	Numerous
M	−	−	0 (<50)	0
N	−	−	0 (<50)	0

markets A–D, *Salmonella invA* was detected by ICAN and standard PCR. From the primary culture of *invA*-positive rinse solution, these four samples showed numerous *Salmonella* on SS agar plates. In samples from markets E–G, *Salmonella invA* was detected by ICAN and standard PCR. A sample from market H showed *invA*-positive in ICAN while negative in PCR. Small number of *Salmonella* colonies (50–950 CFU/ml) was detected in these four samples. In samples from markets I–L, *Salmonella invA* was detected by ICAN but not by standard PCR. Four samples, showed no colonies after primary cultivation but colonies were appeared on the agar inoculated after secondary cultivation. In samples market M and N, two showed no *Salmonella* colonies after both primary and secondary cultivation and these were *invA*-negative in ICAN and PCR.

4. Discussion

In this study, we compared the ICAN and PCR for detection of *invA* of *Salmonella* isolates in Zambia. Our results emphasized ICAN is an efficient and sensitive system to detect *invA* of *Salmonella.* We detected *Salmonella invA* present in chicken carcass rinses by ICAN. ICAN is more sensitive than standard PCR. In the detection, *invA* can be detectable when pure cultures were not used. From the rinse solution, various bacteria were cultured. If the non-*Salmonella* were contaminated, ICAN could detect *invA* and the result completely identified the culture data.

The ICAN could be a valuable tool in screening of samples for important food-borne pathogen like *Salmonella*. It is rapid and cost effective, taking 3 h for performance of the ICAN with a cost of about $15/sample, starting from the DNA extraction to choromato-strip detection. When routine identification method taking several days is used, the cost is more than $18. Standard PCR ($5) is more cost effective than ICAN.

However, in developing countries of Africa, it is difficult to obtain PCR reagents and there have been various machinery troubles (i.e. invasion of sand, dust, and insects). PCR-machine has been immediately bought with the new model. Therefore, final cost of PCR is expensive in developing country. The ICAN is not required PCR-machine. ICAN itself will be cost down in future. Constant temperature was obtained by a water bath. Therefore, ICAN is suitable for survey in developing countries such as Zambia.

Salmonella invA was detected at high rate from chicken carcass rinses by ICAN-choromatostrip. In the positive samples, the number of the bacteria was less than 50 CFU/ml in rinse solution (market H–L). Recently, Hong et al. reported PCR-ELISA for detection of *Salmonella invA* [11]. PCR-ELISA in their study increased sensitivity by 1000-fold (40 CFU/ml) for bacterial cultures and 100-fold (2×10^2 CFU/ml) for *Salmonella* over that of gel-based PCR. In our study, gel-based standard PCR could not detect *Salmonella invA* in samples from the five markets. It is important for not only amplification of nucleic acids but also detection system to research carcass contamination in market level.

Samples from two markets (M and N) were negative for detection of *invA* and culture of *Salmonella*. We concluded these markets were free from *Salmonella*. For public health problem, we must increase markets free from *Salmonella.*

Hinton et al. reported there was a gradation in the invasiveness of *Salmonella* phage type PT4 strains [12]. They also suggest that recent isolates of PT4 may have enhanced virulence for chickens which is not necessarily associated with the carriage of a 38 Md plasmid. Seventy-five percent of PT4 strains in our study showed *invA*-positive. In this phage type, virulence may be different among the strains. Such a specific phage type may originate in the specific animal group bred in the same area.

In this report, *invA* was detected only in 66.7% (egg yolk) and 85.7% (healthy chicken carcass) of *Salmonella* tested. Nolan et al. [13] have suggested that among *invA*, *pagC* and *spvC* genes specific for *Salmonella*, *pagC* is the best choice for use as a probe or PCR target. In their paper, *pagC* was detected 99% of the *Salmonella* used, and *invA* was detected in 94.2% of the isolates. Olah et al. [14] reported that for molecular analysis of *Salmonella*, all isolates (100%) were positive for the presence of *invA* and *pagC* but were negative for *spvC*. In our study, *invA* could be one of the choices for detection of *Salmonella*. In future, several target genes or their products may be targets for detection protocols.

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