

Rapid Detection of *Salmonella* on Commercial Carcasses by Using Isothermal and Chimeric Primer-Initiated Amplification of Nucleic Acids (ICAN)-Enzyme-Linked Immunosorbent Assay (ELISA) in Zambia

Emiko Isogai, DVM¹
Manda Silungwe²
Patson Sinkala²
Carol Chisenga²
Charles Mubita²
Michelo Syakalima, DVM²
Bernard Mudenda Hang'ombe, DVM²

Chitwambi Makungu, DVM²
John Yabe, DVM²
Martin Simuunzab, DVM²
Andrew Nambota, DVM²
Hiroshi Isogai, DVM³
Hideto Fukushi, DVM⁴
Jun Yasuda, DVM⁵

¹*Department of Preventive Dentistry
Health Sciences University of Hokkaido
Hokkaido, Japan*

²*School of Veterinary Medicine
University of Zambia
Lusaka, Zambia*

³*Institute of Animal Experimentation
Sapporo Medical University
Sapporo, Japan*

⁴*Department of Applied Veterinary Sciences
United Graduate School of Veterinary Sciences
Gifu University
Gifu, Japan*

⁵*Veterinary Teaching Hospital
Faculty of Agriculture
Iwate University
Morioka, Japan*

KEY WORDS: *Salmonella*, isothermal and chimeric primer, enzyme-linked immunosorbent assay (ELISA), Zambia

ABSTRACT

Salmonella infections in human population belong to the most important foodborne zoonoses in the world. Therefore, studies on rapid methods for detection of *Salmonella* in animal-derived foods and ready-to-eat-foods are needed. We describe a 2-step method using isothermal and chimeric

primer-initiated amplification of nucleic acids (ICAN) to amplify the *Salmonella invA* and detecting the amplified product using an enzyme-linked immunosorbent assay (ELISA) from rinsed carcass samples in Zambia, Southern Africa. With ICAN-ELISA, *Salmonella* was detected in 20/24 samples (83.3%) from chicken carcass rinses and 4/6 (66.7%) samples from cattle carcass rinses. Contamination of chicken carcasses with *Salmonella* could be associated with the market style in Zambia.

ICAN-ELISA is a rapid and effective approach for the detection and survey of *Salmonella* contamination in markets.

INTRODUCTION

Salmonella species cause a variety of human diseases ranging from gastroenteritis to systemic infection.¹ It has been considered that contamination of carcasses may occur through a chain involving production through consumption.² The introduction of *Salmonella* into the food chain appears to be manifested by early exposure of domestic animals (including poultry) to the organism that results in long-term persistent infections. In Zambia, the detection of salmonellas in chickens, eggs, and cattle is documented.³⁻⁶

Salmonellosis in Zambia has stimulated renewed interest in *Salmonella* and food safety by encompassing meat processors, consumers, and the government. For detection of *Salmonella*, a combination of serologic tests and bacteriologic confirmation has been done in this country. Presently, there is a need for a sensitive non-cultural detection of the pathogen in Zambia. It has been reported that polymerase chain reaction (PCR) for the specific detection of *Salmonella* is evaluated targeting an *invA* gene.⁷ Isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) targeting *invA* has been developed as an innovative method for DNA amplification that is performed at a constant temperature. The detection was performed by an enzyme-linked immunosorbent assay (ELISA). ICAN may offer an alternative method to PCR that requires cycles of different temperature to amplify DNA. The system is a commercially available detecting system without requiring a PCR machine. In this study, we tried ICAN-ELISA to screen *invA* from poultry and beef carcasses of various markets in Zambia. Market style may be one of the risk factors of contamination of the organisms in developing countries such as Zambia.

MATERIALS AND METHODS

The chicken and cattle carcasses were sampled from various markets in Lusaka, Zambia. Prepacked carcasses (frozen) were divided from the largest processing plant in Lusaka. A part of prepacked carcasses (cool) were repacked as a small mass of carcasses. Prepacked and repacked carcasses were under controlled temperature. In contrast, a meat stand without temperature control is a traditional style in Zambia.

Twenty-five grams were sampled aseptically and rinsed in 50 mL phosphate buffer solution (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 *Salmonella Shigella* (SS) agar for 24 hours at 37° C. The identity of suspected *Salmonella* isolates was confirmed by standard method. When primary culture was negative, 1 mL rinse solution was inoculated into 9 mL brain heart infusion (BHI) liquid medium, cultivated for 24 hours at 37° C. The secondary culture was inoculated on SS agar for 24 hours at 37° C.

For DNA extraction from rinse solution, 40 mL was centrifuged at 5000 rpm for 20 minutes; precipitate was re-suspended in 1 mL of distilled water and heated at 95° C for 10 minutes. After centrifugation at 15,000 rpm for 10 minutes, the supernatant was used for DNA extraction.

ICAN was done by using TaKaRa ICAN (*Salmonella* Detection Kit-ELISA 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 minutes in a water bath. The ICAN product was used as a source of amplified *invA*. In the detection of ICAN products by ELISA, the ICAN amplification products (5 µL) plus 50 µL hybridization buffer were added to 2 wells of a streptoavidin-coated 96 well-plate. After incubation for 15 minutes at room temperature, the denaturing reagent (10 µL) was added to each well and incubated for 3 minutes. A detection probe

Table 1. Origin of Carcass, Sale Style of Market, and Detection of Salmonella.

Carcass	Sale Style of Market	Total Examined	Detection of <i>Salmonella invA</i> by ICAN (%)	Positive Number of Primary Culture (%)
Chicken	Prepacked and frozen	9	6 (66.7)	1 (11.1)
	Repacked, cool	11	10 (90.9)	5 (45.5)
	Non packaged and no temperature control	4	4 (100.0)	4 (100.0)
Cattle	Repacked, cool	3	2 (66.7)	1 (33.3)
	Non packaged and no temperature control	2	2 (100.0)	2 (100.0)
	Sausage, cool	1	0	0
Total		30	24 (80.0)	13 (43.3)

or an internal probe (100 μ L) was added to the wells and incubated for 15 minutes. After washing, peroxidase (POD)-conjugated antibody (100 μ L) was added to the wells and incubated for 20 minutes. Substrate solution (TMBZ, 100 μ L) was added after washing. After 10 minutes, a stop solution (100 μ L) was added and optical density (OD) was read with an ELISA reader.

Modified PCR based on the method of Rahn et al⁷ was used for repacked and cooled chicken carcass, to compare with the result of ICAN-ELISA. 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 μ L) were diluted with 8 μ L of PCR buffer containing 10 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, and 0.01% gelatin. The diluted specimens were heated at 94° C for 10 minutes and then kept at 80° C for another 30 minutes. The PCR mixture was directly added to heat-treated specimens so that they contained 0.25 mM each deoxynucleoside triphosphate, 1mM each primer, and 0.5 U of Taq DNA polymerase in a total volume of 20 μ L. 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 ampli-

fied in samples containing strains of *Salmonella* species. The corresponding DNA fragment was not amplified in samples containing the other bacterium such as *Escherichia coli*.

The 5 *Salmonella* strains used as positive control in this study were originated from chicken carcasses in Zambia. The *E. coli* (5 strains) were used as negative controls. These bacteria were cultured in BHI agar for 24 hours at 37° C. A small amount of bacteria (10²–10⁴) was inoculated into distilled water and heated at 95° C for 10 minutes. After centrifugation at 15,000 rpm for 5 minutes, supernatant was used as DNA sample.

RESULTS

We examined 30 carcass rinse samples from 10 areas in Lusaka, Zambia. Table 1 shows *invA*-positive rates using ICAN-ELISA detection. The *invA*-positive chicken carcasses were 66.7% (prepacked and frozen), 90.9% (repacked and cooled), and 100.0% (non-packaged and no temperature control). The *invA*-positive cattle carcasses were 66.7% (prepacked and cooled) and 100.0% (non-packaged and no temperature control). From a salty sausage (cooled), no *Salmonella* was detected. With ICAN-ELISA, *Salmonella* was detected in 20/24 (83.3%) chicken carcass rinses and 4/6 (66.7%) cattle carcass rinses, without the utilization of an enrichment step. From positive samples of ICAN-ELISA, viable

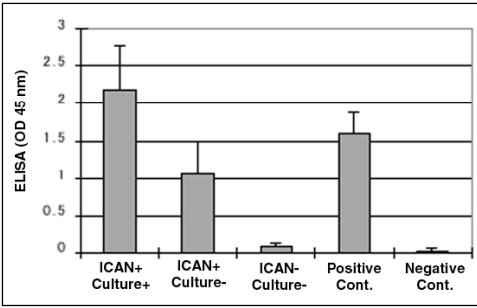


Figure 1. Levels of ICAN-ELISA detection. Culture means primary culture positive. Negative controls: mean \pm standard deviation from 5 strains of *Escherichia coli* (10^6 CFU/mL). Positive controls: mean \pm standard deviation from 5 strains of *Salmonella enteritidis* (10^6 CFU/mL).

Salmonella was isolated from secondary culture, even if the bacterium could not be isolated from primary culture.

Thirteen samples were *Salmonella*-positive in ICAN-ELISA and primary culture (Group 1: ICAN+, Culture+), and 11 samples were *Salmonella*-positive in ICAN-ELISA (Group 2: ICAN+, Culture-). Group 3 (ICAN-, Culture-) showed negative results in both tests. As shown in Figure 1, the OD \pm SD in ICAN-ELISA was 2.190 ± 0.580 in Group 1, 1.059 ± 0.430 in Group 2, and 0.091 ± 0.046 in Group 3. There were significant differences of OD in ICAN-ELISA among the 3 groups (unpaired *t*-test, $P < 0.05$).

Standard PCR showed 5/11 (45.5%) samples from repacked and cooled chicken carcass. These samples also showed positive results of ICAN-ELISA and primary culture. ICAN-ELISA could detect *invA* when standard PCR showed negative results.

DISCUSSION

ICAN-ELISA is a rapid and effective approach for the detection and survey of *Salmonella* contamination in markets. The *invA*-positive rate of prepacked and frozen chickens was 66.7%. The percentage of ICAN-ELISA was higher than that of standard culture method. It has been reported that standard PCR products can be detectable in ELISA with increased sensitiv-

ity by 1,000-fold for bacterial cultures and 100-fold for *Salmonella* over that of gel-based PCR.⁸ Our results showed that a combination method, ICAN and ELISA, is highly sensitive.

Acceptable microbiological quality of ready-to-eat foods is important for food hygiene. Poor microbiological quality in Zambia has been associated with preparation on the premises, premises type, and little or no confidence in the food business management, especially food hygiene and local authority inspector's confidence. Even in such an environment, it is possible to manage temperature control and to avoid human-human cross contamination.

Hollingsworth and Kaplan state that current and future advances applicable to a farm-to-table prevention concept should persist and continually be explored.⁹

The present study has confirmed contamination of chicken carcasses with *Salmonella* in Zambia. This finding could be utilized in further epidemiological studies.

ACKNOWLEDGMENTS

Our study was supported in part by research grant No. 15255021 from the International Scientific Research Program from the Ministry of Education, Science and Culture, Japan.

REFERENCES

- Blaser MJ, Newman LS: A review of human salmonellosis. Infective dose. *Rev Infect Dis* 1982;4:1096-1106.
- Barrow PA: Salmonellosis. Prospects for microbiological control in poultry. *Avian Pathol* 1989;18: 557-561.
- Hang'ombe BM, Sharma RN, Skjerve E, Tuchili LM: Occurrence of *Salmonella enteritidis* in pooled table eggs and market-ready chicken carcasses in Zambia. *Avian Dis* 1999;43:597-599.
- Ngoma M, Pandey GS, Suzuki A, Sato G, Chimana H: Prevalence of salmonella in apparently healthy slaughtered cattle and pigs in Zambia. *Indian J Anim Health* 1996;35:197-200.
- Sato Y, Schneebeli M, Matsukawa K, Chimana H, Sinsungwe H, Sato G: Outbreaks of *Salmonella* Dublin infection among calves on a

- dairy farm applying *Salmonella bacterins* in Zambia. *J Vet Med Sci* 1993;55:511–513.
6. Tuchili L, Ulaya W, Kato Y, Kaneuchi C: Recent characterization of salmonella strains isolated from chickens in Zambia. *J Vet Med Sci* 1996;58:77–78.
 7. Rahn K, Grandis SA, De Clarke RC, et al: Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol Cell Probes* 1992;6:271–279.
 8. Hong Y, Berrang ME, Liu T, et al: Rapid detection of *Campylobacter coli*, *C. jejuni*, and *Salmonella enterica* on poultry carcasses by using PCR-enzyme-linked immunosorbent assay. *Appl Environ Microbiol* 2003;69:3492–3499.
 9. Hollingsworth J, Kaplan B: 1998. Food safety in the United States. In: Kaper JB, O'Brien AD, eds. *Escherichia coli O157:H7 and Other Shiga Toxin-Producing E. coli Strains*. ASM Press: Washington, DC; 1998:109–118.