Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2012, Article ID 264986, 8 pages
doi:10.1155/2012/264986

Research Article

Attenuated *Salmonella typhimurium* SV4089 as a Potential Carrier of Oral DNA Vaccine in Chickens

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Received 23 February 2012; Accepted 10 April 2012

Academic Editor: Chiu-Chung Young

Attenuated *Salmonella* has been used as a carrier for DNA vaccine. However, *in vitro* and *in vivo* studies on the bacteria following transfection of plasmid DNA were poorly studied. In this paper, eukaryotic expression plasmids encoding avian influenza virus (AIV) subtype H5N1 genes, pcDNA3.1/HA, NA, and NP, were transfected into an attenuated *Salmonella enteric typhimurium* SV4089. *In vitro* stability of the transfected plasmids into *Salmonella* were over 90% after 100 generations. The attenuated *Salmonella* were able to invade MCF-7 (1.2%) and MCF-10A (0.5%) human breast cancer cells. Newly hatched specific-pathogen-free (SPF) chicks were inoculated once by oral gavage with 10^9 colony-forming unit (CFU) of the attenuated *Salmonella*. No abnormal clinical signs or deaths were recorded after inoculation. Viable bacteria were detected 3 days after inoculation by plating from spleen, liver, and cecum. Fluorescent *in situ* hybridization (FISH) and polymerase chain reaction (PCR) were carried out for confirmation. *Salmonella* was not detected in blood cultures although serum antibody immune responses to *Salmonella* O antiserum group D1 factor 1, 9, and 12 antigens were observed in all the inoculated chickens after 7 days up to 35 days. Our results showed that live attenuated *S. typhimurium* SV4089 harboring pcDNA3.1/HA, NA, and NP may provide a unique alternative as a carrier for DNA oral vaccine in chickens.

1. Introduction

Avian systemic salmonellosis has three distinct phases and during each phase there is significant interaction with the immune system. The first is invasion via the gastrointestinal tract. The second phase is the establishment of systemic infection mainly as an intracellular infection of macrophages. Finally infection may be cleared by the immune response, the bird may succumb to the infection, or a subclinical carrier state may develop [1].

It has been established that attenuated *Salmonella enterica* serovar Typhi (*S. Typhi*) strains can serve as safe and effective oral vaccines to prevent typhoid fever [2]. In addition, live attenuated *S. typhimurium* strains have been evaluated for use as live vaccines to express or deliver a variety of pathogen's antigen or DNA, respectively, to mucosal lymphoid tissue including the HA of avian influenza virus (AIV) [3], polyprotein of infectious bursal disease virus (IBDV) [4], VapA antigen of *Rhodococcus equi* [5], and 5401 gene of *Eimeria tenella* [6]. In all the above studies, oral administration of the attenuated *Salmonella* may induce immune response with various degrees of protection against the respective pathogens.

The pathogenicity of the bacteria such as *S. typhimurium* can be reduced significantly by various attenuation methods while still retaining their invasion capacity and thus deliver the heterologous genes into mammal cells. *S. typhimurium* SV4089 is a double mutant (Dam^- and $PhoP^-$), derived from wild-type *S. typhimurium* SL1344. This mutant of *Salmonella* did not show pathogenicity to chickens at a dose level as high as 10^{10} CFU/mL delivered orally [4, 6]. The oral LD50 determined for chicken, of wild-type SL1344, is about 10^4 CFU [7]. Studies have shown that Dam methylation regulates other virulence-related loci besides spv genes in the virulence plasmid such as LPS modification genes, *Salmonella* pathogenicity island 1, and the std fimbrial

Table 1: Primers used for amplification of HA, NA, and NP of A/Ck/Malaysia/5858/04 (H5N1) in pcDNA3.1 vectors.

Primer	Sequence (5′ to 3′)	Gene	Tm (°C)
HAF	ccc caa GCT TAT GGA GAA AAT AGT GCT T	HA	58.5
HAR	ccc gga GGA TCC AAT GCA AAT TCT GCA TTG TAA	HA	63.2
NAF	ccc caa CAA GCT TAT GAA TCC AAA TAA GAA	NA	57.5
NAR	ccc gaa TTC CTT GTC AAT GGT GAA TGG	NA	59.7
NPF	aaa aag CTT ATG GCG TCT CAA GGC ACC	NP	59.7
NPR	ggg gga TCC ATT GTC ATA TTC CTC TGC	NP	61.3

Extra six nucleotide bases in lower case were added before recognition sites to facilitate amplification.

operon are under Dam methylation control [8, 9], whilst PhoP-PhoQ is regulatory system that controls expression of several virulence properties in *S. typhimurium* [10]. In addition, the virulence possessions that the PhoP$^-$ PhoQ system controls include the ability to survive inside macrophages, withstanding acidic pH, invasion of epithelial cells, confrontation of killing by antimicrobial peptides, formation of spacious vacuoles, and the ability to alter antigen presentation [11].

Although *S. typhimurium* has successfully been used as a carrier for vaccine, characteristics of the bacteria and its *in vitro* and *in vivo* stability in chickens are poorly studied. Hence, the purpose of this study is to characterize the attenuated *S. typhimurium* SV4089 transfected with eukaryotic expression plasmid encoding AIV genes.

2. Materials and Methods

2.1. Attenuated Salmonella, Media, and Reagents. Attenuated *Salmonella enterica sv.* Typhimurium (*S. typhimurium*) SV4089 was kindly provided by Dr. Josep Casadesus (Department of Molecular Genetics University of Sevilla Spain). The bacteria is a double mutant (*dam*$^-$*228*::Mu*dJ* (Dam$^-$) *phoP7953*::Tn*10* (PhoP$^-$)), derived from wild-type *S. typhimurium* SL1344 [4, 6]. Bacterial strains were cultured in Luria Bertani (LB) broth or agar. The eukaryotic expression plasmids, pcDNA3.1 (Invitrogen, USA) expressing AIV subtype H5N1 A/Ck/Malaysia/5858/04, HA, NA, and NP genes (pcDNA3.1/HA, NA, and NP) were constructed by Jalilian et al. [12]. The nucleotide sequences of the full length of HA, NA, and NP are available at GenBank under accession number DQ320934, DQ321066, and DQ321132, respectively.

2.2. Transformation of S. typhimurium. A single colony of attenuated *S. typhimurium* SV4089 was grown in LB broth to an optimal density OD600 0.6–0.8 and resuspended in ice-cold Nuclease-Free Water. The plasmids pcDNA3.1/HA, NA, NP and control plasmid (pcDNA3.1) were purified (Qiagen, Germany) and transformed into *S. typhimurium* competent cells by electroporation set at 2.5 kV, 25 μF, and 200–400 Ω (Gene Pulser, Bio-Rad, USA). The transformed culture (100 μL) was suspended into LB plates supplemented with 50 μg/mL ampicillin [4, 6]. Resistant colonies harboring the plasmid were cultured and stored after confirmation by PCR and FISH to the targeted AIV HA, NA, and NP.

2.3. Stability of Plasmids in S. typhimurium. To determine the *in vitro* stability of the transfected plasmid in a population of attenuated *S. typhimurium*, bacterial cultures were passaged for approximately 100 generations (25 days) without antibiotic selection. The percentage stability of the plasmid was estimated by calculating the number of cells containing the plasmid at each passage divided by the number of *Salmonella*.

2.4. Invasion Assay of Salmonella on Eukaryotic Cells. The human breast cancer cell lines, MCF-7 and MCF-10A, were maintained in Dulbecco's Modified Eagles Medium (DMEM) with 10% (v/v) fetal calf serum and 10% (v/v) antibiotic and antimycotic (GIBCO, Invitrogen, USA) then infected with *S. typhimurium* as described by Cano et al. [13] with modification. Briefly, 10^6 CFU of bacteria were added to the cell culture at 60 to 70% confluency in 24-well plates with each well containing 10^5 cell, multiplicity of 10 : 1 infection (bacterium/eukaryotic cell ratio). After incubation for 1 h, extracellular bacteria were killed by incubation for 2 h at 37°C in DMEM containing 100 μg of gentamicin/mL. Cells were washed three times in phosphate-buffered saline (PBS), pH 7.4, and lysed with 1% Triton X-100-PBS (pH 7.4) for 5 min at room temperature. Viable counts of the intracellular bacteria in the lysate were made on LB agar. All samples were run at least in duplicates.

2.5. PCR Amplification of Targeted AIV Genes. A set of primers were designed based on the consensus sequences for amplification of HA, NA, and NP genes of A/Ck/Malaysia/5858/04 (H5N1) (Table 1). Amplification was facilitated by an additional 6 bp to the 5′ end of the primer. Gene fragments of interest were amplified by gradient PCR using a PCR Thermal Cycler (MJ Research, PTC-225). Briefly, in a 50 μL volume, 25 μL dH_2O was mixed with 1.5 μL of 10X reaction PCR buffer, 0.6 μL of 50 mM $MgCl_2$, 0.6 μL of dNTP mix (10 mM each), 10 μL each of 100 mM forward and reverse primers, 0.3 μL Taq DNA polymerase (5 U/μL) (Vivantis, Malaysia), and 2 μL (50 ng) template. The amplification was performed in a PCR thermal cycler using the following parameters: one cycle of 94°C for 5 min; 30 cycles of 94°C for 1 min, 56.9°C for 1 min, 72°C for 2 min,

Table 2: Specific labeled probes used for the detection of AIV genes.

Sequence (5′ to 3′)	Gene
Cy5 atg GAG AAA ATA GTG CTT CTT TTT GCA ATA	HA
Alexa 350 atg AAT CCA AAT AAG AAG ATA ATA ACC ATC	NA
Fluo atg GCG TCT CAA GGC ACC AAA CGA TCT TAT	NP

To facilitate binding of probe and labeling with dye, an additional 3 bp (atg) were added to the 5′ end of the primer.

final extension step of 72°C for 10 min, and a hold cycle at 4°C.

2.6. PCR Detection of Attenuated S. typhimurium. PCR assays were conducted based on the detection of Random Amplified Polymorphic DNA (RAPD) as described by Gurakan et al. [14]. Briefly, in a 50 μL reaction, 27 μL dH_2O was mixed with 10 μL (50 ng) template, 0.6 μL of 50 mM $MgCl_2$; 1.5 μL of 10X reaction PCR buffer; 0.6 μL 0f 10 mM deoxynucleoside triphosphate; 0.3 U of *Taq* DNA polymerase (Vivantis, Malaysia); 10 μL of 10 mM primer 5′-CGT GCA CGC-3′ [14]. The reaction mixture was denaturated at 90°C for 5 min followed by 35 × [89°C, 1 min; 32°C, 1 min; 72°C, 1.5 min], an extension cycle at 50°C for 3 min, and a hold cycle at 4°C. The PCR product and loading dye were mixed and separated on 1% agarose gel with 1X TAE buffer (Promega, Germany) at 80 V for 40 min.

2.7. FISH Detection of Transfected Plasmid and Salmonella. The targeted HA, NA, and NP genes in transfected *Salmonella* were detected using specifically designed and labeled HA, NA, and NP probes (Table 2) by FISH. Meanwhile, a specific probe for the 23S rRNA of *Salmonella* was used, Sal3 (5′-AATCACTTCACCTACGTG-3′) [15, 16] to detect the attenuated *S. typhimurium*. The probe was synthesized and labeled with Cy5 at the 5′ end (NextGene, Germany).

Briefly, *Salmonella* and *Salmonella*/pcDNA3.1/HA, NA, and NP were fixed and hybridized using the protocol described by Tabatabaei et al. [17] with some modification. The cells (2 mL) were fixed in 6 mL of 3% paraformaldehyde/phosphate buffer saline. The fixed cell suspensions were diluted with ethanol/PBS mixture (1 : 1) and were spotted on coated glass slides (Cel-Line, New Hampshire, UK). Bacterial smears were covered with 20 μL of lysozyme, incubated for 20 min at 30°C. The slides were thoroughly rinsed with double-distilled water followed by air-drying at room temperature for the termination of enzymatic digestion.

Samples in 8 μL of hybridization buffer and 1 μL of Sal3, HA, NA, and NP probe were separately applied to the wells on the slides and incubated. Unbound probes were removed by rinsing the slide in 1 mL of washing solution. The slides were then incubated at 48°C for 20 min in 50 mL of washing solution, rinsed briefly with distilled water, air-dried, and mounted with SlowFade antifading reagent (Molecular Probes, OR, USA). Fluorescence was observed using confocal laser microscopy (MRC 1024ES, BioRad, USA).

2.8. Preparation of Salmonella Inocula. The inocula were prepared from typical *Salmonella* (clear with black center on XLT4) colonies of an overnight culture, which were transferred 3 times in LB medium. The bacterial cells were collected by centrifugation at 5000 ×g for 10 min and serially diluted in sterile Buffered Peptone Water (BPW) (pH 7.2) to a concentration of approximately 10^9 CFU/mL.

2.9. Bacteriological Analysis of SPF Chickens. Eighteen-day-old SPF eggs were purchased from Malaysian Vaccines and Pharmaceuticals and reared under *Salmonella*-free environment. Cloacal swabs from one-day-old SPF chicks were directly preenriched in BPW at 37°C for 24 h, after which the samples were enriched by the addition of 1 mL of this suspension to 9 mL of Rappaport-Vassiliadis 10 broth (AES Lab, India). Following the incubation at 42°C for 20 h, 100 μL of this suspension was inoculated on XLT4 (AES Lab, India) plates with novobiocin (25 μg/mL)/nalidixic acid (20 μg/mL) (NO-NA) of culture media and incubated for 20 h at 37°C.

2.10. Inoculation of SPF Chickens with Attenuated Salmonella. Newly hatched chickens were subjected to oral inoculation with attenuated *Salmonella* using an autoclaved gavage plastic tube and a 1 mL syringe. The inocula were drawn through the gavage plastic tube, which was then dipped in glycerol for a smoother passageway down the chicken esophagus. A dose of 500 μL containing approximately 10^9 cells was administered orally to each chick. Chickens were monitored daily for clinical changes and were provided free access to water and a balanced unmedicated corn-soybean-meal-based mash layer diet that met or exceeded the National Research Council (NRC) requirements for nutrients. The experimental trials were approved by the animal care and use committee at the Faculty of Veterinary Medicine, Universiti Putra Malaysia, UPM/FPV/PS/3.2.1.551/AUP-R72.

2.11. Clinical and Bacteriologic Surveillance. During the period of clinical observation, four chickens at day 3, 5, 7, 10, 14, 21, 28, and 35 were euthanized for sample collection. Cecum, liver, heart, and spleen were collected in sterile condition and homogenized, and 10-fold dilutions were made in BPW starting from 10 to 10,000. From each dilution, 100 μL samples were cultured on NO-NA XLT4 plates. The number of CFU/g of tissue was determined by counting the bacterial colonies. Five colonies of bacteria were picked randomly at each time point for PCR identification. Multiple blood cultures were also obtained after inoculation to detect bacteremia. Freshly collected blood were directly preenriched

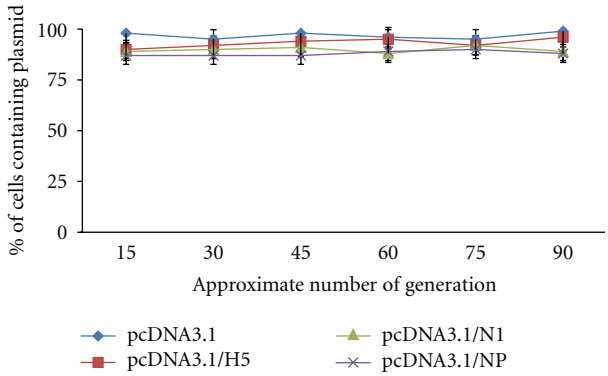

Figure 1: *In vitro* stability of pcDNA3.1/HA, NA, NP, and pcDNA3.1. Recombinant *S. typhimurium* containing transfected plasmids were passaged for approximately 100 generations. The percentage of bacteria containing the plasmid was determined by viable count on media without the appropriate antibiotic selection. No significant difference was recorded ($P > 0.05$).

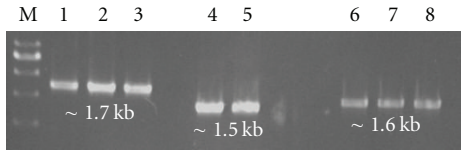

Figure 2: PCR amplification of HA, NA and NP, genes of pcDNA3.1 vector from transfected *S. typhimurium*. M = 100 bp marker (Fermentas, Germany) lane 1–3 = HA; 4-5 = NA; 6–8 = NP.

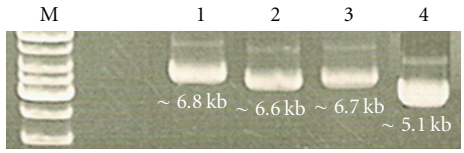

Figure 3: Extracted circular plasmids from transfected *Salmonella*: M = 1 kb marker (Fermentas, Germany); 1 = pcDNA3.1/HA; 2 = pcDNA3.1/NA; 3 = pcDNA3.1/NP; 4 = pcDNA3.1.

and enriched with BPW and Rappaport-Vassiliadis 10 broth (AES Lab, India). One milliliter of this culture was then plated on NO-NA XLT4 and incubated for 20 h at 37°C, and examined for growth. Blood was collected for serum slide agglutination of antibodies to *Salmonella* O antiserum group D1 (factors 1, 9, 12) (Difco, Becton Dickinson, USA).

3. Results

3.1. Characterization and Antibiotic Resistance of Salmonella Colony on XLT4. Plating results of *S. typhimurium* SV4089 on XLT4 provide good presumptive morphologic evidence for the presence of *Salmonella* species. Colonies on XLT4 plate typically are large, black with a creamy margin (after 30 h) and circular, with convex to umbonate/nippled elevations and entire margins. Both the nontransfected as well as pcDNA3.1, pcDNA3.1/HA, pcDNA3.1/NA, and pcDNA3.1/NP transfected attenuated *S. typhimurium* SV4089 are NO-NA resistant.

3.2. In Vitro Invasion Assay of Attenuated Salmonella into Mammalian Cells. The average invasion rate obtained in standard 60 min infection was 1.2% ± 0.09 in MCF-7 and 0.5% ± 0.08 in MCF-10A cells.

3.3. In Vitro Stability of pcDNA3.1/HA, NA, NP, and pcDNA3.1 in S. typhimurium. S. typhimurium SV4089 transfected with pcDNA3.1/HA, NA, NP and pcDNA3.1 were grown without antibiotic and each point represents the percentage of bacterial population that has retained the plasmid. The plasmid was stably detected over 90% of the attenuated *S. typhimurium* population after 100 generations of growth in antibiotic-free media (Figure 1).

3.4. PCR Screening of HA, NA, and NP, for Salmonella after Transfection. PCR screening of the transfected *Salmonella* showed that pcDNA3.1/HA, pcDNA3.1/NA and pcDNA3.1/NP from transfected *Salmonella* were successfully transformed into attenuated *S. typhimurium* cells separately. The viral HA, NA and NP genes of A/Ck/Malaysia/5858/04 (H5N1) virus were amplified from the respective transfected *Salmonella* by PCR. Figures 2 and 3 showed the expected sizes of the amplified product for HA (1.7 kb), NA (1.5 kb), NP (1.6 kb) and circular pcDNA3.1 (5.1 kb), pcDNA3.1/HA (6.8 kb), pcDNA3.1/NA (6.6 kb), pcDNA3.1/NP (6.72 kb), respectively.

3.5. Clinical and Bacteriologic Results. Cloacal swabs were collected from one-day-old SPF chickens. No *Salmonella*-positive chickens were found. To test the possibility of colonization of *S. typhimurium* SV4089 into organs, chickens were infected orally with a dose of approximately 10^9 cells. *Salmonella* was found in the spleen, liver, and cecum of the infected birds after 3 days of infection.

The systemic counts of *Salmonella*-positive peaked at 7 days after infection in all the selected organs following which they declined at day 14, where no bacteria were detected in those organs although *Salmonella* showed good stability in cecum until day 35 (data not shown). No *Salmonella* growth was detected in the heart. As expected, the control group was *Salmonella*-free throughout the experiment. Antibody against *Salmonella* was detected by adding one drop of *Salmonella* O antisera group D1 with one drop of the collected serum on a slide. Although, *Salmonella* were not detected in blood cultures, sera from inoculated chickens from as early as 7 days of vaccination up to 35 days were detected positive for *Salmonella*-specific antibodies.

3.6. FISH of the Transfected Plasmid and Salmonella. Under optimal hybridization conditions, transfected HA, NA and NP genes into *Salmonella* were specifically visualized and detected using the corresponding probes (Figure 4). Meanwhile, *Salmonella* was specifically detected using genus-specific probe, Sal3 from homogenized spleen, liver and

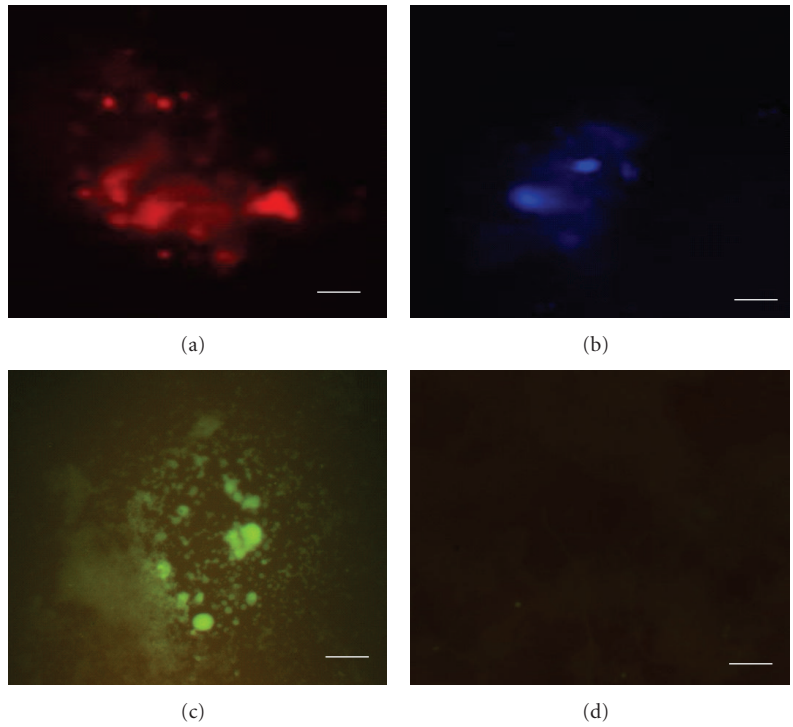

Figure 4: FISH detection of HA, NA and NP. (a) pcDNA3.1/HA, (b) pcDNA3.1/NA, (c) pcDNA3.1/NP, (d) pcDNA3.1 as negative control after transfected into *S. typhimurium* strain SV4089 by confocal laser microscopy. Probes were labeled with Cy5 (red), Alexa 350 (blue) and Fluo (green) fluorescent dye, respectively. The white bar in each image represents 2 μm.

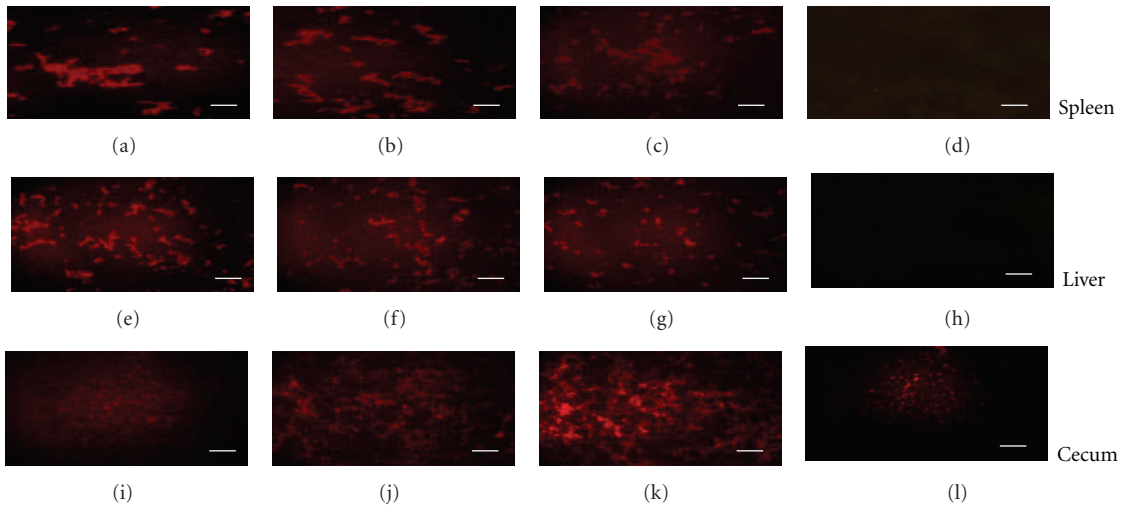

Figure 5: FISH detection of *Salmonella* from infected homogenized spleen after (a) 3 days, (b) 7 days, (c) 10 days, (d) negative control, liver after (e) 3 days, (f) 7 days, (g) 10 days, (h) negative control, cecum after (i) 3 days, (j) 7 days, (k) 21 day, (l) 35 days infection. The white bar in each image represents 2 μm.

cecum sections of infected chicken, whereby a distinct fluorescent signal from rod-shaped bacteria could be detected following hybridization (Figure 5). *Salmonella* was detected from infected organs (spleen, liver and cecum) 3 days after inoculation. After two weeks, the infected chickens showed good clearance of *Salmonella* from spleen and liver, while *Salmonella* were detected in cecum even at 35 days after inoculation.

3.7. PCR of Salmonella . Detection of *Salmonella* using PCR was performed using genus-specific primers for rapid screening of pure culture and extracted *Salmonella* from tissue.

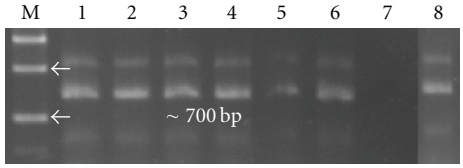

Figure 6: Amplification results of *S. typhimurium* from homogenized infected organs; 1-2 = spleen and liver after 7 days; 3-4 = spleen and liver after 10 days; 5-6 = cecum after 10 and 35 days; 7 = negative control; 8 = positive control. M = 1 kb marker (Fermentas, Germany). Detection of 700 bp amplification product was found as specific polymorphic marker for *S. typhimurium*. Arrows indicate 500 bp and 1,000 bp DNA fragments.

As shown in Figure 6, the RAPD patterns contained three different bands, in which the size varied between 300 and 1,000 bp. The presence of 700 bp amplification product was found as specific polymorphic region for *S. typhimurium*.

4. Discussion

A major disadvantage of DNA vaccine is its low efficiency, which is inherent in the way the vaccine is currently being administered. In addition, parenteral administration of DNA vaccine is not practical in livestock animals including poultry that requires mass vaccination. Thus, it is essential to develop carrier systems that improve efficacy of genetic vaccines. Live bacteria that contain recombinant plasmids encoding heterologous antigen genes from other pathogens have the potential as oral delivery vectors for DNA vaccines. The pathogenicity of bacteria such as *S. typhimurium* could be decreased significantly by various attenuation methods while still retaining their invasion capacity to deliver the heterologous genes into mammalian cells [13]. A few studies in chickens have shown that *S. typhimurium* harboring heterologous genes were capable of eliciting specific humoral and cellular immune responses locally and at the systemic level [4, 6]. However, characterization studies on attenuated *Salmonella* as DNA vaccine carrier in chicken is limited.

In this study, the characteristics of the attenuated *S. typhimurium* SV4089/harboring pcDNA3.1, pcDNA3.1/HA, NA, and NP gene were investigated. First, the transfected bacteria were genetically stable *in vitro* when passing on LB agar with or without antibiotic selection as well as on XLT4 agar. Secondly, PCR, FISH, and culturing on XLT4 were able to detect *Salmonella* from homogenized spleen, liver, and cecum. No chickens displayed any clinical aberrations, side effects, and abnormalities after inoculation with the attenuated *Salmonella* at dose of 10^9 CFU within 5 weeks after oral inoculation. Previous studies have shown that the attenuated bacteria are not pathogenic to chickens at dose levels as high as 10^{10} CFU/mL delivered orally [4, 6]. However, the virulent strain of the *Salmonella* SL1344 has an oral LD50 of 10^4.

Salmonella colonize the small intestine and invade normally nonphagocytic epithelial cells in order to gain access to the underlying lymph tissue. Invasion is mediated by a type III secretion system (T3SS) encoded on *Salmonella* pathogenicity island 1 (SPI1). The SPI1 T3SS forms a needle-like complex that is responsible for the injection of bacterial effector proteins into the host cell cytosol [18]. Another important hallmark of *Salmonella* pathogenesis is resistance to antimicrobial attack of phagocytic cell [13]. These mechanisms enable serovar *Typhimurium* to efficiently go through the intestinal epithelial barrier and distribute to deeper tissues. The lack of PhoP-PhoQ two-component system has always been linked to major intramacrophage survival defects and marked attenuation in the murine typhoid model. Hence, human cancer cell lines are commonly used to study invasion of *Salmonella* to eukaryotic cells [19]. The attenuated *Salmonella* used in this study still maintained its ability to invade MCF-7 and MCF-10A mammalian cells, where 1.2% and 0.5% of the cells were infected, respectively. In addition, *in vitro* study of *Salmonella* serovar Enteritidis phage type 4, strain 76Sa88 showed invasion into T84 human colonic adenocarcinoma cell line up to 5.75% [20]. Meanwhile, the average invasion rate of *Salmonella enterica* serovar Typhimurium strains into HeLa cells and NRK-49F rat fibroblasts was up to 1-2% and 0.2–0.5%, respectively [13].

In order to enhance the specificity and sensitivity of *S. typhimurium* SV4089 detection among the other *Enterobacteriaceae* in the gastrointestinal tract, XLT4 media is uniquely formulated with surfactant, tergitol [21]. In this study, XLT4 media supplemented with NO-NA could serve as a selective medium for *S. typhimurium* SV4089. In addition, we showed that initial resuscitation of *Salmonella* in selective medium with NO-NA followed by other detection methods such as PCR and FISH is effective to avoid any-false positive results. Previously, Gürakan et al. [14] have used RAPD analysis to detect *S. typhimurium* from other selected *Enterobacteriaceae* and showed specific band for the detection of *S. typhimurium* is 700 bp. Using similar approach, a 700 bp band was detected in smear and infected organs of *S. typhimurium* SV4089.

The safety of a live vaccine and stability of an expression plasmid in a vaccine vector may potentially affect the efficacy of the vaccine and alter the outcome of vaccination. One of the limitations of using bacteria especially *Salmonella* as a carrier is monitoring the stability and availability of bacteria without any cultivation. Our study demonstrated that 5′ monolabeled DNA oligonucleotide is able to detect plasmid DNA in prokaryotes without signal amplification-based or target amplification by *in situ* RT-PCR (Figure 4). The fact that FISH can detect nonculturable bacteria is an advantage. A 23S rRNA FISH method, using Sal3 probe was used as a rapid and direct screening tool on attenuated *S. typhimurium* SV4089 in pure culture and tissue homogenates from chickens inoculated with *Salmonella* (Figure 5). The method allowed us to detect the bacteria from tissues of the inoculated chickens within approximately 7 h prior to the culture of *Salmonella* (necessary time for fixation, hybridization, and observation of the sample) [22–24]. In this study, rod-shaped *Salmonella* was detected from homogenized spleen, liver, and cecum as early as 3 days after inoculation. The *Salmonella* count peaked at day 7 although at day 14 no bacteria were detected in those organs except from cecum. Previous study has shown that *S. enteritidis* aroA invades

phagocytes of the liver, spleen, and bone marrow [25]. In addition, extensive bacterial multiplication occurred in the ceca. Clearance of *S. typhimurium* from the gastrointestinal tract occurred considerably later than the clearance from the spleen and liver [26, 27]. The results presented here indicate that although attenuated *S. typhimurium* SV4089 is able to move through different organs of chicken, it is not able to cause septicemia. In addition, sera from the inoculated chickens react strongly with O antiserum group D1 indicating the attenuated *Salmonella* is a suitable vaccine candidate for oral vaccination.

Salmonella has been used as live vehicles for inducing protective responses to a wide variety of diseases since due to its ability to improve vaccine efficacy by targeting vaccines *in vivo* inductive sites on mucosal surface or internal organs. The growth of the live bacteria can also be controlled by common antibiotics. In addition, *Salmonella* naturally possesses LPS that can function as an adjuvant to stimulate systemic immune responses [28].

In conclusion, live attenuated *Salmonella* serovar Typhimurium provides a unique alternative in terms of *in vitro* stability of transfected plasmid, *in vivo* stability, relatively inexpensive to produce in large scale, and easy detection by acting as a carrier in chickens.

Acknowledgments

This paper was supported by project no. 06-05-ABI-AB002, Agro-Biotechnology Institute, Ministry of Science, Technology and Innovations and HICOE grant, Ministry of Higher Education, Government of Malaysia. The author would like to thank Dr. Meisam Tabatabaei for his assistance in FISH assay.

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