

◀Research Note▶

Experimental Infection of Egg-laying Hens with *Salmonella enterica* Serovar Enteritidis Phage Type 4 and its Three Mutants

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The emergence of *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) during the past three decades as major contaminant of eggs and other poultry products caused a surge in human infections. This could have been mediated in part by emerging *S. Enteritidis* strains with enhanced virulence. The overall pathogenicity of *Salmonella* is controlled by numerous genes. To assess the role of a few specific genes thought to contribute to the pathogenicity of *S. Enteritidis* in egg laying hens, we conducted an experimental infection of egg-laying hens, with a wild type (WT) *S. Enteritidis* phage type 4 strain and three mutants (M1, M2, M3). These mutants were produced from the wild type by the inactivation of the *prgH*, *SEN4287*, and *tyrR* genes, respectively. We observed that the WT and the M1 mutant had shorter durations of fecal shedding and faster clearance from internal organs of the infected hens than the M2 and M3 mutants. The isolation rates of the wild type *S. Enteritidis* and the mutants were highest from the ceca, moderately high from the liver and spleen, and lowest from the ovaries of the infected hens. Hep-2 cells attachment assay revealed attenuated attachment for the M1 mutant while the M3 mutant seemed to have enhanced attachment. Colonization of tissues of the infected hens by M1 mutant appears to have been attenuated.

Key words: egg-laying hens, experimental infection, mutants, *Salmonella* Enteritidis, pathogenicity

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Introduction

Salmonella enterica serovar Enteritidis (*S. Enteritidis*) has emerged as a common cause of foodborne disease for the past three decades. While the general number of *S. Enteritidis* cases has declined, the number of outbreaks has remained relatively stable. Consumption of eggs, egg products, and chicken meat, are the recognized major risk factors for *S. Enteritidis* infection in the US. (St Louis *et al.*, 1988; Hedberg *et al.*, 1993; Kimura *et al.*, 2004). *Salmonella* are estimated to account for 1.4 million cases of illness and over 500 deaths a year in the United States (Mead *et al.*, 1999). Economic losses caused by these infections approaches up to \$2.3 billion per year in this country (Frenzen *et al.*, 1999). Recent estimates indicated

that egg-borne *S. Enteritidis* infections range from 81,535 to 276,500 in the United States during 2000 (Schroeder *et al.*, 2005).

Transovarian infection in hens, leading to egg contamination has been a major suspected contributor to the emergence and spread of *S. Enteritidis* in eggs (Thiagarajan *et al.*, 1994; Guard-Petter, 2001; Velge *et al.*, 2005; Gantois *et al.*, 2009). However, specific virulence factors that may be associated with the emergences of this pathogen have not been well elucidated. It is conceivable that enhanced pathogenicity of certain *S. Enteritidis* strains to egg laying hens may have contributed to their emergence as significant contaminants of eggs and other poultry products.

Recently, several genes associated with contamination of chicken eggs and oviduct colonization were identified (Clavijo *et al.*, 2006; Gantois *et al.*, 2008; Li *et al.*, 2009). The role of numerous genes, which are involved in cell wall structural and functional integrity and nucleic acid and amino acid metabolism, were assessed in the survival of *S. Enteritidis* in egg albumin in vitro (Clavijo *et al.*, 2006). Twenty-five genes induced in *S. Enteritidis* during oviduct colonization and egg contamination in laying hens were also identified, among which 7 were isolated from both the oviducts and the contaminated eggs and involved

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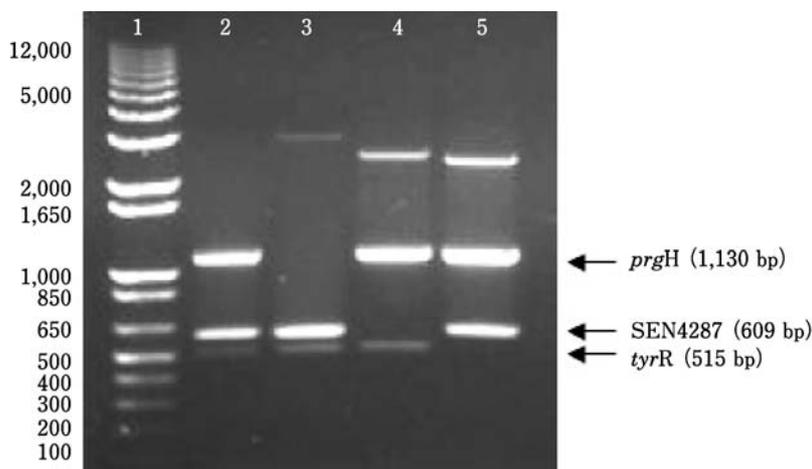


Fig. 1. **Validation of mutants with inactivated genes.** Multiplex PCR was performed using primer sets for 3 genes (*prgH*-F: GCGCTCTCAGCTTTTGACTT and *prgH*-R: TTCCTTACTGGTATCCTACT; SEN4287-F: AGCAGCCAAAAGAGGTGTTG and SEN4287-R: GGTTCGCGGTATCCCATAAT; *tyrR*-F: CGGCGAAGATCACGAAAT and *tyrR*-R: TATGCGATACCCCAACC). Mutants harbor a Tn insertion (1.8 kb)
 Lane 1: DNA marker (1Kb Plus DNA Ladder™, invitrogen)
 Lane 2: Wild type
 Lane 3: M1 (inactivation of *prgH* gene in wild type; expected size of band: 2.93 kb)
 Lane 4: M2 (inactivation of SEN4287 gene in wild type; expected size of band: 2.41 kb)
 Lane 5: M3 (inactivation of *tyrR* gene in wild type; expected size of band: 2.32 kb)

in amino acid and nucleic acid metabolism, motility, cell wall integrity, and stress responses (Gantois *et al.*, 2008).

To understand the role of specific genes associated with egg contamination in the pathogenicity of *S. Enteritidis*, this study was carried out to determine if the 3 selected genes responsible for survival of *S. Enteritidis* in egg albumin under an *in vitro* assay may contribute to the overall pathogenicity of *S. Enteritidis* in experimentally infected egg-laying hens. We inoculated several groups of egg-laying hens with a wild type (WT) *S. Enteritidis* phage type 4 strain and three mutants designated as M1, M2, and M3. These mutants were produced from the wild type strain by using transposon-mediated mutagenesis for the inactivation of *prgH*, SEN4287, and *tyrR* genes, respectively (Clavijo *et al.*, 2006). We have compared the mutants to the wild type strain for infectivity, colonization, and persistence, in egg laying hens to reveal any significant modulation of the pathogenicity related to the mutated genes. We have also analyzed the wild type and the mutants, for their interaction patterns with Hep-2 cells maintained in tissue culture.

Materials and Methods

Bacterial Strains

A highly virulent *S. Enteritidis*, phage type 4 strain (SE 2427, LD₅₀ in mice = 15 organisms), isolated from human outbreaks (Lu *et al.*, 1999) was selected as a wild type (WT) strain. Three mutants were generated from the WT using transposon-mediated mutagenesis. All three mutants harbor a Kanamycin resistance gene (1.4 kb) carried by a transposon, which disturbed the normal function of each

target gene (Fig. 1). In mutant 1 (M1) the region of the *prgH* gene (a component of the type III secretion apparatus), in mutant 2 (M2) the region of the SEN4287 gene (*S. Enteritidis* specific-possible restriction endonuclease gene, and in mutant 3 (M3) the region of the *tyrR* gene (a regulator of aromatic amino acid biosynthesis and transport) were disrupted as previously described (Clavijo *et al.*, 2006).

Experimental Infection of Egg Laying Hens

Forty white Leghorn egg-laying hens (approximately 45 week old) of the commercial strain “Highline W36” were purchased from a vendor in Michigan. All birds were housed in individual standard wire mesh cages at the University infectious disease containment facility in compliance with the USDA/NIH requirements as previously described (Lindell *et al.*, 1994). All birds were tested for *Salmonella* infection by fecal and egg culturing prior to experimental infection. All hens were labeled with leg-bands and randomly divided into four isolated groups of ten hens each. Cultures of the four *S. Enteritidis* strains (WT, M1, M2, and M3) were grown overnight at 37°C in Brain Heart Infusion Broth (Oxoid LTD., Basingstoke, Hampshire, England). Nine chickens of each group were orally inoculated by gavaging each bird with approximately 10⁹ CFU/ml of the respective *S. Enteritidis* strain suspended in 0.5 ml of sterile PBS using a tuberculin syringe and a curved, bulbed 10 cm needle, as previously described. (Lindell *et al.*, 1994). All hens were monitored for fecal shedding and egg contamination with the inoculated *S. Enteritidis* strains. Colonization of and clearance of the organisms from internal organs were monitored

by sacrificing 4 birds from each group 2 weeks after the inoculation. The remaining 20 birds were monitored for fecal shedding for an additional 2 weeks.

Bacteriological Analysis

Xylose Lysine Tergitol 4 (XLT-4) agar containing 100 mg/L of Kanamycin was used to select for the growth of the *S. Enteritidis* mutants based on the presence of the Kanamycin resistance gene insertion. XLT-4 without Kanamycin was used for wild type isolation. Eggs were collected twice a week and cultured for *S. Enteritidis* isolation on the same day of collection. Before bacteriologic culturing, eggs from each bird were soaked for 5 seconds in a boiling water bath to kill surface microbial contaminants, dried and cracked. The contents of 2–3 eggs per bird were pooled in a sterile Whirl-Pak bag (Nasco, Fort Atkinson, WI, USA) and homogenized with 25 ml of Buffered Peptone Water (BPW) as a primary enrichment, and incubated at 37°C for 72 hours. One milliliter of the primary BPW enrichment was transferred to tetrathionate broth as a secondary enrichment and incubated for 24 hours at 37°C, then plated on XLT-4 agar. After 48 hours of incubation at 37°C, suspect colonies were transferred to individual Triple Sugar Iron agar slants. Positive samples were confirmed by serotyping with *Salmonella* O antiserum (group D1) using slide agglutination.

Ten grams of mixed fecal material were taken from a 3-day fecal output collected using a disposable tray beneath each caged hen. Fecal samples were placed in sterile Whirl-Pak bags, weighed and 10 ml of tetrathionate broth was added per gram of fecal material. Each bag was mixed with a Stomacher lab blender (Tekmar, Cincinnati, OH, USA) before incubation at 37°C for 24 hr. To determine the intensity of fecal shedding, a quantitative analysis of the *Salmonella* in each hen's fecal samples was performed. One milliliter from each stomached fecal sample was serially diluted and inoculated onto XLT-4 plates using the Autoplate 4000 (Spiral Biotech, Norwood, MA, USA) and incubated for 24 hours at 37°C before counting the colonies with the Q-Count camera and software (Spiral Biotech, Norwood, MA, USA). Samples that did not produce colonies with the spiral plating technique were inoculated on XLT-4 agar plates after overnight incubation in tetrathionate enrichment broth media to qualitatively assess the presence or absence of *Salmonella*. In order to assess organ colonization, four of the nine birds from each group were necropsied two weeks after the infection. Birds were euthanized by placing them in a CO₂ chamber, followed by cervical dislocation. The carcasses were disinfected by dipping in a 2% Benzalkonium chloride solution. Birds were posted and inspected for gross pathology. Tissue samples were collected using aseptic technique. Organs, including ovarian tissue, cecal tonsil, liver and spleen, were collected for bacteriological and histological analyses. Pieces of these organs were placed in 10% phosphate buffered formalin and were submitted to the Diagnostic Center for Population Animal Health at Michigan State University for histopathological analysis.

Qualitative analysis of viable *Salmonella* organisms in the internal organs (liver, spleen, and ovarian tissue) was performed similarly to the fecal analysis. Four weeks after the infection, the remaining five hens from each group were necropsied and samples were processed as described for the first groups.

Wild type and the 3 mutants isolated from fecal and internal organ sources were tested for attachment to Hep-2 cells as previously described (Thiagarajan *et al.*, 1994). Hep-2 cells were obtained from ATCC and were maintained in MEM tissue culture medium according to ATCC instructions.

Serological Analysis

For the measurement of serum antibody titer, ELISA method was used as previously described (Saeed *et al.*, 1999). Serum samples were serially diluted in PBS Tween-20, pH 7.4 (Sigma Chemical Co, St. Louis, MO, USA). The 96-well plate was sensitized with 2 µg per well of *S. Enteritidis*-specific lipopolysaccharide (Sigma) dissolved in carbonate buffer (pH 9.6). The blocking agent used was 100 µl of 1% Bovine Serum Albumin in PBS tween-20 which was applied to each well of the plate and the plate was incubated at 37°C for 30 min. After washing with PBS tween-20, the diluted serum samples were applied to the ELISA plate and incubated at 37°C for 45 min. After washing with PBS tween-20, 50 µl of anti-chicken IgY conjugate (Sigma) solution (1:1000 dilution of PBS tween-20) was applied to the ELISA plate and incubated at 37°C for 45 min. Fifty microliters of colorimetric substrate solution of p-Nitrophenyl Phosphate enzyme solution (Sigma) was added to each well of the ELISA plate and the plate was incubated at 37°C for 30 min. The colorimetric reaction was stopped with 3 N NaOH. Using a spectrophotometric plate reader, the optical density of each well was measured at an absorbance of 405 nm. End point titers of the serum samples were determined by calculating a mean OD and standard deviation of the ELISA readings at 6 different dilutions from control birds. The ODs were compared at six different dilutions and positive end point was defined by looking into the highest dilution associated with a positive OD, which is larger than the mean value plus two standard deviations than the baseline at that dilution. For each dilution, geometric mean titer was calculated using natural logarithm.

Quality Control

To verify the identity of the mutants in the cultures of fecal and tissue samples, samples were inoculated onto XLT-4 containing Kanamycin. We also performed a growth comparison with the mutant strains plated on XLT-4 media versus the Kanamycin containing XLT-4 to ensure that there was no significant difference in growth due to the presence of the Kanamycin. Each of the four *S. Enteritidis* strains used in experimental infection was stored at –80°C. After the necropsy, the *S. Enteritidis* isolates from all positive organ samples from the infected chickens during the 4 week period post inoculation were further evaluated using Multi-locus Variable Number

Tandem Repeat Analysis (MLVA) as previously described (Cho *et al.*, 2007) and compared with the original stored *S. Enteritidis* strains to ensure freedom of cross contamination among the infected birds.

Data Analysis

To test for an association between positive bacteriological results and hens from each group, the chi-square test (or Fisher's exact test) and t-tests were used. In addition, quantitative assessment, expressed in CFU/gm of fecal samples, duration of fecal shedding, and *S. Enteritidis* prevalence in tissues of each cultured organ were calculated to provide data on the degree of infectivity for each tested *Salmonella* strain. All statistical analyses for comparisons were performed using PC SAS system for Windows version 9.1 (SAS Institute, Cary NC). Comparisons with p-values <0.05 were considered statistically significant.

Results and Discussion

All baseline fecal and egg samples of the 40 hens were negative for *S. Enteritidis* by bacteriological culture. Morphological examination of *S. Enteritidis* colonies from cultured fecal samples of infected birds revealed that on XLT4-plated medium, mutant strain M1 consistently produced smaller colonies than those of the wild type strain and other mutants (data not shown). While we do not have an exact explanation for this finding, it is conceivable that the disrupted region of the *prgH* gene that encodes for a component of the type III secretion apparatus in the wild type (Clavijo *et al.*, 2006) may have some influence on the colonial size of the *S. Enteritidis* or the ability of the mutant to grow at the normal rate on selective media such as XLT4.

S. Enteritidis was isolated from most of the cultured fecal samples (Fig. 2) but from none of the egg samples (~5 eggs pools/bird/week) in qualitative testing during the study period. Bacterial colony counts in fecal samples from all inoculated birds were high during the first few

days post-infection but decreased during the last 2 weeks of the experiment (Fig. 2). Hens inoculated with mutant strains M3 ($p < 0.05$) and M2 had a longer duration of fecal shedding than the wild type and M1 strains.

In all of the birds, the isolation rates of *S. Enteritidis* were the highest from the cecal samples. Isolation from liver and spleen was moderate while lower isolation was observed in cultured ovaries (Fig. 3). Hens inoculated with WT and M1 strains had faster clearance of the organisms from internal organs than hens that were inoculated with M2 and M3 mutants (Fig. 3a and 3b). In general, it was noted that isolation of *S. Enteritidis* from all internal organs was more common at 2 weeks than at 4 weeks post-infection. However, M2 ($p < 0.05$) and M3 were readily isolated from liver and spleen by the 4 weeks of the experiment compared to the wild type, which was cleared, from liver and spleen within 4 weeks. These findings may suggest a delayed clearance of M2 and M3 strains from the body systems in comparison with the wild type. Despite isolation of the wild type *S. Enteritidis* and its 3 mutant strains from the ovarian tissue, negative egg cultures may be due to the low number of bacteria in the ovarian tissue which was not enough to contaminate eggs. All mutants and WT were cleared from ovarian tissue within 4 weeks which is consistent with reported observations on the ovarian immune response to *Salmonella* infection in birds (Lillehoj *et al.*, 2007).

Examination of stained ovarian tissue sections, made from necropsied hens 2 weeks post infection, revealed that ovarian tissue from hens inoculated with M3 mutant showed more intense colonization of the pre-ovulatory follicles than hens inoculated with WT, M1, and M2 strains. Such observation on increased persistence of this mutant in the ovarian tissue may be due to failure to elicit a stronger cellular immune response required for its clearance from this site (Lillehoj *et al.*, 2007). Additionally, it could be due to repeated systemic infection with the mutant that persisted in the cecal site for a longer time

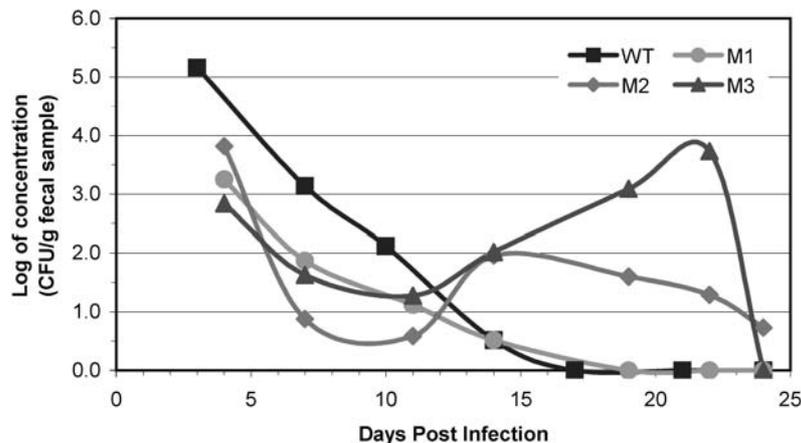


Fig. 2. Intensity and duration of fecal shedding in hens experimentally infected with wild type and the 3 mutants of *S. Enteritidis* phage type 4.

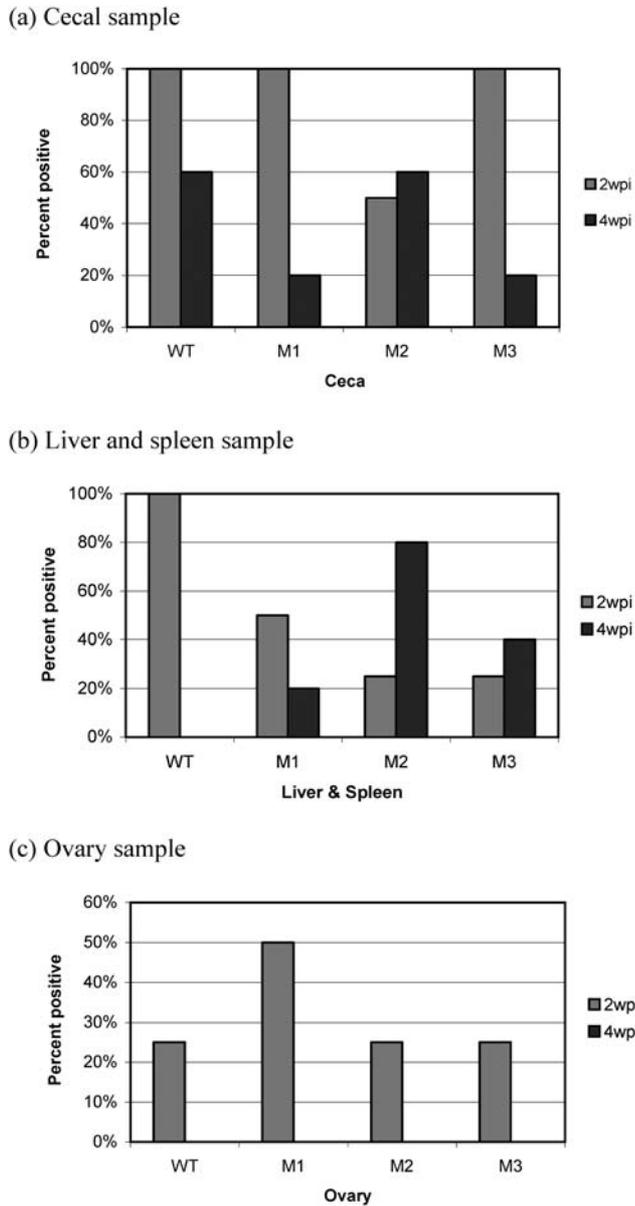


Fig. 3. Isolation rate of *S. Enteritidis* wild type and its three mutants from different sites of hens experimentally infected with the respective strains during the 4 weeks follow-up.

- (a) Cecal sample
 (b) Liver and spleen sample
 (c) Ovary sample

than other strains. However, these observations need additional investigation.

The underlying factors for the patterns of interaction between the mutant strains and bird system in each group of hens may be a reflection of a complex interplay; the M2 mutant has a Tn insertion in a gene (SEN4287, homologous to a restriction endonuclease) unique to *Salmonella* serovar Enteritidis and the gene is assumed to be associated with increased survival in egg albumen (Clavijo

et al., 2006). The M3 mutant resulted from the inactivation of the metabolic regulator (*tyrR* gene), which could be associated with alteration of the recognizable surface structures on the bacteria. Therefore, it is conceivable that inactivation of *tyrR* gene in *Salmonella* Enteritidis may help the bacteria to evade the cellular immune response and organ clearance in experimentally-infected egg laying hens (Andrews *et al.*, 1991; Clavijo *et al.*, 2006).

We have found that the attachment pattern and intensity of the M1 mutant to Hep-2 tissue culture cells was lower than that of the wild type and the other 2 mutants. This observation suggests that inactivation of the *prgH* gene, which is located on the chromosome of *Salmonella* pathogenicity island I (SPI-I), disrupted the function of a structural component formation of type III secretion needle complex (Kubori *et al.*, 1998) and may have reduced the bacterial attachment to Hep-2 cells. Recently, it was reported that SPI-I encoded T3SS mediate the intimate contact of *Salmonella* Typhimurium with non-phagocytic cells (Lara-Tejero and Galan, 2009). Therefore, it is possible to attribute the attenuation of the interaction of M1 with hen tissue system as well as attachment to Hep-2 cells to the inactivation of the gene.

In attachment assays, patterns of mannose resistant local attachment (MRLA) were exhibited by all strains that demonstrated the ability to attach to Hep-2 cells in vitro. In comparison to the WT strain, attachment assays using Hep-2 cells, revealed attenuated attachment for the M1 mutant (attached to less than 20 percent of the Hep-2 cells), while the M3 mutant seemed to have enhanced attachment (attached to more than 60 percent of the examined Hep-2 cells). The M3 mutant harbors a gene (*tyrR*) disrupted by a Tn insertion, which appears to be involved predominantly in the metabolism of aromatic amino acids (Clavijo *et al.*, 2006). However it is not clear how inactivation of this gene leads to an increased persistence of the mutant in hen tissues and its enhanced attachment to Hep-2 cells.

The ELISA-based serological testing demonstrated a slight fluctuation in the LPS-specific serum titers of hens infected with the WT and the 3 mutants (differences were not statistically significant). This finding suggests that the mutational process did not result in significant differences in the immune response to the 3 mutants as measured by using LPS-specific antibody detection ELISA (Fig. 4).

In this study, the M1 mutant (with inactivation of *prgH* gene) was expected to be less virulent than wild type, based on the notion that a change in cell wall structure/or function (a structural component of type III secretion apparatus) may be associated with peculiar colonial morphology and attenuated attachment to Hep-2 cells as well as low colonization/infectivity of chickens. This study suggests that even though alteration of a few specific genes may not be sufficient to significantly attenuate the overall pathogenicity to experimentally-infected laying hens, inactivation of *prgH* gene may have led to measurable change in the interaction between the mutant and the

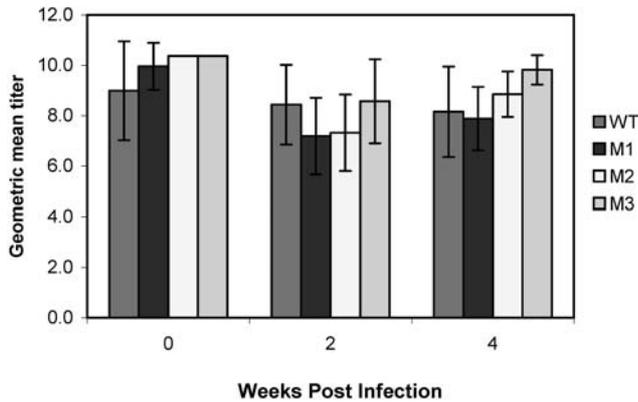


Fig. 4. Patterns of serological immune response measured by ELISA in hens experimentally-infected with wt and 3 mutants during the 4 weeks of follow-up post infection.

infected hens. Further investigation using larger samples of hens and longer observation periods may be needed to more accurately assess the role of these genes in the pathogenesis of *Salmonella* Enteritidis in laying hens.

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