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Development and evaluation of live attenuated *Salmonella* vaccines in newly hatched duckings

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ABSTRACT

Domestic ducks remain a major source of zoonotic *Salmonella enterica* infections for man worldwide and approaches to protection should include vaccine-mediated immunity. With this in mind we developed several genetically defined mutants in a virulent duck *Salmonella typhimurium* isolate TT-1. From initial tests for virulence in day-old ducks, Δ*rpoS*, Δ*hilA*, and Δ*slyA* mutants retained some virulence so were not studied further. Amongst the mutants showing greater attenuation, Δ*ssrB*, Δ*phoPQ*, Δ*ompR*, and Δ*clpP* also showed high levels of protection when 1-day-old ducks, which were vaccinated orally, were challenged 1 week later demonstrating the capacity to protect ducks in the first few weeks of life when they are most susceptible and when the risk of infection is greatest. Immunized ducks triggered Omp-specific IgG, IgM, and IgA responses and raised IL-2 and IFN-γ levels in the serum coupled with IL-4 suppression.

1. Introduction

Although duck rearing for meat and eggs is a relatively minor component of the poultry industry in western countries, duck meat has traditionally been a major source of animal protein in many Asian countries. World production was approximately 4.4 million tons in 2013 and may exceed 4.5 million tons in 2015. Production increased by 40% between 2000 and 2010, greater than the increase in production of other poultry species with the corresponding increases in Asia and particularly China being 44% and 47% respectively. The estimated production in China is 2.76 million tons representing 82% or production in Asia (www.fao.org; www.thepoultrysite.com).

Food-borne zoonotic pathogens are thus of increasing interest to the duck industry, some of which, such as *Salmonella*, may additionally cause considerable economic losses [1]. Isolation of *Salmonella* serovars from ducks has been recorded from many countries and recently from Egypt [2], South Korea [3], Malaya [4], Vietnam [5] and less recently from the United Kingdom [6]. Isolation has been reported from 22 of the 28 Chinese provinces and regions since 1981 [7–13] with isolation from raw duck meat reaching 69% in some cases [14]. Given the general absence of hygienic controls associated with duck rearing it is not surprising that a variety of serovars are frequently isolated [2,15].

Compared with the available information on *Salmonella* infection in domestic fowl our knowledge of the course of infection in ducks is much poorer [16]. This paucity of information includes approaches to control for a species of poultry where hygienic limitations are much greater than with chickens. The possibilities of using competitive exclusion flora have not been explored extensively although live vaccines administered orally can induce a similar effect by virtue of their ability to colonize the intestine [17]. Antibiotic therapy results in resistance in *Salmonella*; levels of resistance are variable but can be high with a tendency toward multi-resistance [2,18] in some cases with strains resistant to 16 or more antimicrobials [3,10,13,15].

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Vaccination has been tested in ducks and although *S. Enteritidis aroA* mutant was found not to be protective the immunity generated by infection with a wild-type *S. Enteritidis* indicates that vaccination should be possible and clearly a practical option. There are unpublished reports of effective protection using commercially available vaccines developed for use with chickens (see [16]). In chickens live attenuated *Salmonella* vaccines generally confer better protection than killed vaccines [19], because the former stimulate both cell-mediated and humoral immunity [20]. To date, many genetically defined live attenuated *Salmonella* vaccines have been reported for use with chickens but not assessed for their efficacy in ducks.

Auxotrophic strains, especially those conferred by mutations in the aromatic pathway [21,22] or in purine biosynthesis [23,24], are attenuated and effective live vaccines. Mutants with defective virulence determinants may also be considered as candidate vaccines. These include a key *Salmonella* Pathogenicity Island 1 (SPI1) regulator *hilA* mutation which reduces invasive ability and virulence when inoculated intragastrically [25,26] and defective SPI2 mutants such as *ssrB* [27,28]. In addition, null mutations in genes expressing global virulence regulators such as PhoP/PhoQ, OmpR, SlyA, ClpP, RpoS and Fis also induce significant reductions in virulence [29–39]. Some of these mutations have been found to be attenuating and protective in chickens [40]. Of the mutations mentioned above none has been tested thus far for their attenuating effects in ducks and neither has their potential protective ability.

We have produced several genetically defined mutants of wild type strain TT-1, a highly virulent *S. typhimurium* strain isolated from the carcass of a young duck in Henan province, China. Their attenuation and safety, immunogenicity and protection against a lethal challenge were assessed in young ducks.

2. Materials and methods

2.1. Bacterial strains, media, and standard genetic manipulations

All bacterial strains used in this study are listed in Table 1. Strains TH4702 (LT2 pKD46) and TH6701 (LT2 Δ*ara*BAD925::*tetRA*) were kind gifts from Prof. Kelly T. Hughes at the University of Utah. Cells were routinely grown in Luria–Bertani (LB) broth. Antibiotics were added to LB agar or broth at the following final concentrations: 100 μg/ml ampicillin for PKD46-containing strains and tetracycline at 15 μg/ml. The generalized transducing phage of P22*HT*105/1 *int*-201 was used in all transductional crosses [19,41].

2.2. Construction of tetRA insertion mutants and markless deletions

To generate strain TT-2 (TT-1 pKD46), P22 phage lysates of TH4702 were used to transduce pKD46 [42] into TT-1, selecting for ampicillin resistance.

The construction of *tetRA* insertion mutants and markerless deletions followed protocols described previously [43]. The resulting insertion (TT-1 to TT-9) and deletion (TT-11 to TT-16) derivatives are shown in Table 1. Strain TH6701 was used as template for PCR amplifying *tetRA*. All primers are listed in supplementary material.

2.3. Measurement of in vitro growth kinetics

To evaluate the *in vitro* growth rate of mutant strains, overnight broth cultures were diluted to an OD600 of ~0.05 using fresh trypticase soy broth (TSB) and incubated at 37 °C at 180 rev/min. At hourly intervals 1 ml samples were taken and the OD measured. The experiments were done in triplicate.

2.4. Virulence assay for Salmonella TT-1 and isogenic derivatives

1-Day-old Sichuan ducks were purchased from a local commercial hatchery, and housed in cages. Prior to the experiment, all birds were screened for *Salmonella* maternal antibody (SMA) using an indirect ELISA described below and only SMA-free birds were used. To determine the 50% lethal dose (LD_{50}) of the parent strain TT-1 in 1-day-old ducks, 50 birds were divided randomly into 5 groups, and dosed orally with 0.2 ml aliquots of *Salmonella* TT-1 ranging from 10^6 CFU to 10^{10} CFU. The control group was dosed with the same volume of diluent (phosphate-buffered saline containing 0.01% gelatin, BSG, [44]). The method of Reed and Muench method was used to calculate the LD_{50} [45]. To evaluate the virulence of isogenic derivatives, the oral inoculation dose of each mutant was adjusted to the concentration of 10-fold and 100-fold the LD_{50} value for TT-1. The number of birds which died or were

Table 1
Bacterial strains, virulence and protection.

Strain	Genotype	Source	Virulence of wild type and mutant strains under different doses (No. of survived/total)		Protection (No. of survived/total) after different vaccinating doses	
			1.2×10^9 CFU	1.2×10^{10} CFU	1.2×10^6 CFU	1.2×10^8 CFU
S. typhimurium						
TH4702	LT2 pKD46	Hughes KT	–/–	–/–	–/–	–/–
TH6701	LT2 Δ*araBAD*925::*tetRA*	Hughes KT	–/–	–/–	–/–	–/–
TT-1	Wild-type	This study	–/–	0/10	–/–	–/–
TT-2	TT-1 pKD46	This study	–/–	–/–	–/–	–/–
TT-3	TT-1 Δ*hilA*::*tetRA*	This study	–/–	–/–	–/–	–/–
TT-4	TT-1 Δ*ssrB*::*tetRA*	This study	–/–	–/–	–/–	–/–
TT-5	TT-1 Δ*phoPQ*::*tetRA*	This study	–/–	–/–	–/–	–/–
TT-6	TT-1 Δ*ompR*::*tetRA*	This study	–/–	–/–	–/–	–/–
TT-7	TT-1 Δ*rpoS*::*tetRA*	This study	–/–	–/–	–/–	–/–
TT-8	TT-1 Δ*slyA*::*tetRA*	This study	–/–	–/–	–/–	–/–
TT-9	TT-1 Δ*clpP*::*tetRA*	This study	–/–	–/–	–/–	–/–
TT-10	TT-1 Δ*hilA*	This study	7/10	4/10	–/–	–/–
TT-11	TT-1 Δ*ssrB*	This study	10/10	10/10	20/20	17/20
TT-12	TT-1 Δ*phoPQ*	This study	10/10	10/10	20/20	20/20
TT-13	TT-1 Δ*ompR*	This study	10/10	10/10	20/20	20/20
TT-14	TT-1 Δ*rpoS*	This study	5/10	1/10	–/–	–/–
TT-15	TT-1 Δ*slyA*	This study	9/10	6/10	–/–	–/–
T-16	TT-1 Δ*clpP*	This study	10/10	10/10	20/20	16/20
					Non-immunized birds	8/20

¨–/–M̆eans not tested.

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