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Evaluation of the immunogenicity and safety of the paratyphoid component in a novel tetravalent attenuated freeze-dried vaccine for pigs

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Abstract

This study evaluated the safety and immunogenicity of the paratyphoid (*Salmonella choleraesuis*) component in a novel tetravalent attenuated freeze-dried vaccine (V4AFD) designed to protect against pasteurellosis, erysipelas, classical swine fever, and paratyphoid in pigs. The attenuated *S. choleraesuis* strain (Smith W.H.) was used for vaccine production, while a virulent strain (S2) served for challenge experiments. Trials were conducted on guinea pigs and pigs. The minimum protective dose (MPD) of the paratyphoid component conferring 100% protection in pigs was determined as 2×10^9 CFU/dose. The vaccine demonstrated good safety, with no adverse reactions observed in pigs administered 10 doses or guinea pigs given two doses. For efficacy, 21 d post-vaccination, 100% (15/15) of vaccinated pigs exhibited positive antibody responses via ELISA and achieved 100% protection (15/15) against challenge with *S. choleraesuis* S2, whereas 100% (3/3) of control pigs succumbed or showed typical lesions. Protective antibodies persisted at high levels, with 100% (10/10) of pigs remaining ELISA-positive after 6 months. These findings confirm that the paratyphoid component in V4AFD is safe, induces strong and sustained protective immunity, and is unaffected by other antigenic components, highlighting its potential for integrated swine disease control programs.

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Introduction

Paratyphoid fever (*salmonellosis*) in pigs, caused primarily by *Salmonella* enterica serovar *Choleraesuis*, represents a significant zoonotic and economic threat to the global swine industry. This pathogen induces systemic infections, including septicemia, pneumonia, meningitis, and high mortality rates in post-weaned pigs[1,2]. Other serovars, such as *S. typhimurium*, contribute to foodborne illnesses in humans through cross-contamination in the food chain[3,4]. In Vietnam, paratyphoid, alongside pasteurellosis (*Pasteurella multocida*), erysipelas (*Erysipelothrix rhusiopathiae*), and classical swine fever (*Pestivirus*), are classified as 'red diseases', necessitating stringent control measures[5]. Vaccination remains the most effective and sustainable strategy for reducing disease incidence, pathogen shedding, and environmental contamination[5,6]. However, intensive swine farming faces challenges from monovalent or bivalent vaccines (e.g., *pasteurellosis*-paratyphoid), which require multiple administrations, escalating labor costs, material expenses, and animal stress. Such stress can transiently impair immunity, reduce productivity, and increase susceptibility to secondary infections[7].

The development of polyvalent vaccines, combining multiple antigens in a single dose, addresses these issues and aligns with modern veterinary vaccine technology trends. A novel tetravalent attenuated freeze-dried vaccine (V4AFD) targeting the four aforementioned diseases has been developed and pilot-produced. Despite advantages, polyvalent vaccines risk antigenic competition, where multiple antigens may diminish immune responses to individual components[8,9]. Recent advancements in *Salmonella* vaccines emphasize live-attenuated strains for eliciting both humoral and cell-mediated immunity, crucial for clearing intracellular pathogens like *Salmonella*[10,11]. Contemporary studies have explored recombinant live-attenuated vaccines[12], oral delivery methods[13], and bivalent formulations against *S. choleraesuis* and *S. typhimurium*[14], demonstrating reduced clinical signs and shedding. Longitudinal investigations also highlight the benefits of sow and piglet vaccination in lowering *Salmonella* prevalence[15].

Materials and methods

Vaccine, bacterial strains, and experimental animals

This study aimed to rigorously assess the safety, immunogenicity, and duration of immunity of the paratyphoid component (*S. choleraesuis*) in V4AFD using guinea pig and pig models, ensuring no antigenic interference from other components. The V4AFD vaccine, produced by the Central Veterinary Institute incorporated attenuated strains for pasteurellosis, erysipelas, classical swine fever, and paratyphoid (*S. choleraesuis* Smith W.H.), following established protocols for live-attenuated vaccine production[12,16]. S2, isolated from Vietnamese outbreaks, represents local epidemiology[5]. The virulent *S. choleraesuis* S2 strain was used for challenges, sourced from a reference collection, and confirmed for pathogenicity via standard virulence assays (LD50 assays in mice and pigs, with > 90% mortality at 10^9 CFU)[17]. Experiments involved 79 healthy guinea pigs (300–350 g) and 107 Landrace × Yorkshire pigs (20–30 kg), all seronegative for the target diseases and housed under controlled conditions with ethical approval from the institutional animal care committee, in compliance with international guidelines for animal experimentation[18,19]. Growing pigs were chosen as a standard model per OIE[20], facilitating controlled trials; while weaned piglets are primary targets, age-related efficacy variations are minimal in analogous vaccines[15]. Animals were acclimatized for one week prior to trials, with daily health monitoring to ensure baseline welfare.

Determination of minimum protective dose (MPD)

Vaccine dilutions yielded paratyphoid concentrations of 1×10^9, 2×10^9, and 3×10^9 CFU/dose, prepared using sterile phosphate-buffered saline (PBS) and verified by colony counting on selective media[11]. Dose gradients were selected based on references to similar attenuated *Salmonella* vaccines[12,16], and preliminary trials showing suboptimal protection below 1×10^9 CFU. Guinea pigs (n = 5/group) received 1/5 pig doses (2×10^8, 4×10^8, 6×10^8 CFU) intramuscularly, while pigs (n = 5/group) received full doses. Vaccinations were administered under aseptic conditions, following standard veterinary practices[6]. After 21 d, animals and controls (n = 3 pigs, n = 2 guinea pigs/batch) were challenged intravenously with S2 at a dose calibrated to induce consistent morbidity in unvaccinated subjects[21]. Protection rates were monitored for 10 d, including clinical scoring for symptoms such as fever, anorexia, and lethargy, with necropsy performed on deceased animals to confirm *Salmonella*-specific lesions[17].

Safety evaluation

Safety was assessed per Vietnamese standard TCVN 8685-1:2011, supplemented by international benchmarks[20], using three vaccine batches with ≥ MPD. Pigs (n = 2/batch) received 10 doses intramuscularly, and guinea pigs (n = 5/batch) received two doses. Animals were observed for local (e.g., swelling, redness) and systemic reactions (e.g., fever, behavioral changes) over 10 d, with vital signs recorded twice daily[16]. Reactions were graded on a 0–4 scale[20]: fever defined as rectal temperature > 40 °C; local swelling measured by caliper and graded as 0 (none) to 4 (> 5 cm severe); systemic signs (e.g., lethargy, anorexia) based on daily clinical scoring.

Efficacy evaluation

Efficacy of three V4AFD batches was compared to a bivalent pasteurellosis-paratyphoid vaccine (positive control) and unvaccinated controls (negative). Intravenous challenge was used for reproducibility[21]; oral routes may yield milder disease but comparable protection[11]. Pigs (n = 5/batch) received one dose; controls (n = 3) received none. At 21 d: (i) Challenge: pigs were intravenously challenged with 1 MLD (3×10^9 CFU) of S2 and monitored for 10 d, including bacterial reisolation from organs[10]. Efficacy required ≥ 80% protection in vaccinated groups and 100% mortality/severe disease in controls. (ii) Antibody Detection: sera were tested using PrioCHECK® *Salmonella* Ab porcine 2.0 ELISA kit (positive if percentage positivity [PP] ≥ 40%), validated against gold-standard methods[6]. Histopathology used H&E staining, scored 0–3 for lesions (e.g., necrosis); bacterial recovery via culture on selective media. Samples were processed in duplicate to ensure reproducibility.

Duration of immunity

Pigs (n = 10) received one V4AFD dose; controls (n = 5) received none. Sera were collected at day 0, 21, 3 months, and 6 months for ELISA analysis, with storage at −80 °C to preserve integrity[15].

Statistical analysis

Data were analyzed using Epicalc 1.02 and Microsoft Excel 2010. Data normality checked via Shapiro-Wilk; ANOVA with post hoc LSD tests compared means; differences were significant at $p < 0.05$. Survival analysis employed Kaplan-Meier curves for challenge outcomes. Group sizes were based on regulatory minima and power calculations for 80% detection of differences ($p < 0.05$).

Results

MPD determination

Among five growth media tested, CT4 (BHI + 1.2% agar + 0.5% glucose + 0.5% beef extract + 2% peptone + 5% rabbit blood) yielded the highest *S. Choleraesuis* biomass ($80.00 \pm 0.16 \times 10^9$ CFU/mL; $p < 0.05$ compared to other formulations) and was selected for production, demonstrating superior nutrient optimization for bacterial propagation. Guinea pig trials showed 100% protection at $\geq 4 \times 10^8$ CFU (equivalent to 2×10^9 CFU/pig dose), with no clinical signs or mortality in protected groups, while lower doses resulted in partial morbidity. Pig validation confirmed MPD as 2×10^9 CFU/dose, with 100% protection at this and higher levels, evidenced by the absence of fever, normal feed intake, and no pathological lesions upon necropsy. In contrast, at 1×10^9 CFU, only 60% survival was observed across batches, with surviving pigs showing mild septicemia symptoms resolving within 7 d, and deceased animals exhibiting classic *Salmonella* pathology such as hepatic necrosis and splenomegaly (Table 1). Controls exhibited 33%–67% mortality, with rapid onset of severe symptoms within 48 h post-challenge, underscoring the virulence of S2. Statistical analysis revealed significant differences in survival rates between MPD and sub-MPD groups ($p < 0.01$).

Safety

All pigs (2/2 per batch) at 10 doses and guinea pigs (5/5 per batch) at 2 doses remained healthy, with no reactions observed, including zero incidence of local inflammation or systemic fever across all monitoring

Table 1. MPD evaluation of paratyphoid component in pig's post-challenge.

Batch	PTH concentration (CFU/dose)	Pigs vaccinated (n)	Challenge dose (MLD)	Survivors (n)	Diseased (n)	Deaths (n)	Evaluation
Batch 1	1×10^9	5	1	3	2	2	Fail
Batch 1	2×10^9	5	1	5	0	0	Pass
Batch 1	3×10^9	5	1	5	0	0	Pass
Control	–	3	1	1	2	2	–
Batch 2	1×10^9	5	1	3	2	2	Fail
Batch 2	2×10^9	5	1	5	0	0	Pass
Batch 2	3×10^9	5	1	5	0	0	Pass
Control	–	3	1	1	2	2	–
Batch 3	1×10^9	5	1	3	2	2	Fail
Batch 3	2×10^9	5	1	5	0	0	Pass
Batch 3	3×10^9	5	1	5	0	0	Pass
Control	–	3	1	1	2	2	–

points. Vital signs remained stable, and no behavioral anomalies were noted, confirming batch consistency (Table 2).

Efficacy

ELISA: 100% (15/15 V4AFD; 5/5 bivalent) pigs seroconverted (PP ≥ 40%) at 21 d, with mean PP values ranging from 65% to 85%, indicating strong humoral responses; controls (0/3) remained negative with PP < 10% (Table 3). Challenge: 100% protection in vaccinated groups (15/15 V4AFD; 5/5 bivalent), with no bacterial recovery from organs and normal histopathology; 100% controls (3/3) died or showed lesions, including pulmonary consolidation and enteric inflammation, with high bacterial loads (Table 4). Kaplan-Meier analysis showed significant survival divergence ($p < 0.001$).

Duration of immunity

100% (10/10) vaccinated pigs maintained positive ELISA responses through 6 months, with mean PP values of 75% ± 8% at 21 d, 60% ± 7% at 3 months, and 55% ± 6% at 6 months, all above the ≥ 40% protective threshold (Table 5). No waning was observed, with intra-group variability minimal (SD < 10%).

Discussion

Determining the MPD (2 × 10^9 CFU/dose) provides a scientific foundation for vaccine formulation, ensuring optimal protection without excess antigen load, which could otherwise lead to unnecessary production costs or potential reactogenicity. The efficacy plateau at ≥ 2 × 10^9 CFU aligns with dose-response patterns in similar vaccines[14], and higher doses were not explored due to regulatory alignment and ethical considerations. The Smith W.H. strain's attenuation maintains immunogenicity while eliminating virulence, consistent with historical data[16] and recent recombinant approaches that incorporate genetic modifications for enhanced safety and efficacy[12]. This MPD aligns with doses reported in bivalent vaccines, where similar bacterial loads have achieved comparable protection against heterologous challenges[14].

Safety results affirm V4AFD's compliance with national and international standards, with no reactions at overdose, aligning with live-attenuated *Salmonella* vaccines' profiles that emphasize minimal residual virulence[6,20]. The absence of adverse events in both species underscores the vaccine's suitability for field use, particularly in stress-sensitive intensive farming systems, where vaccine-induced stress could exacerbate disease susceptibility[7]. While short-term safety was confirmed, future field trials should include long-term histopathology to rule out immunopathology. Comparative studies on monophasic *Salmonella* variants have similarly reported high safety margins for commercial vaccines like *Enterisol Salmonella* T/C, which reduce colonization without side effects[22].

Efficacy data demonstrate robust humoral responses and full protection, equivalent to bivalent vaccines, indicating no antigenic competition despite the polyvalent nature[8,9]. This is noteworthy, as polyvalent vaccines can sometimes compromise responses due to immune resource allocation, but here, the integration of four antigens succeeded, possibly owing to an optimized formulation that allows balanced antigen presentation. Live-attenuated strains elicit comprehensive immunity, including cell-mediated responses critical for intracellular pathogen clearance[10,11], corroborated by recent bivalent oral vaccines reducing clinical disease and shedding in challenged pigs[14]. Furthermore, the 100% seroconversion and protection rates surpass those in some longitudinal field studies, where environmental factors may dilute efficacy[15].

Table 2. Safety evaluation of V4AFD.

Batch	Animal	Dose (per animal)	Survivors/vaccinated (*n*)
1	Guinea pig	2	5/5
1	Pig	10	2/2
2	Guinea pig	2	5/5
2	Pig	10	2/2
3	Guinea pig	2	5/5
3	Pig	10	2/2

Table 3. ELISA efficacy at 21 d post-vaccination.

Group	Pigs (*n*)	ELISA positive/vaccinated (*n*)	Positive (%)
V4AFD batch 1	5	5/5	100
V4AFD batch 2	5	5/5	100
V4AFD batch 3	5	5/5	100
Bivalent control	5	5/5	100
Unvaccinated control	3	0/3	0

Table 4. Challenge efficacy.

Group	Animal	Vaccine dose	Survivors/challenged (*n*)	Protection (%)
V4AFD batch 1	Pig	1	5/5	100
V4AFD batch 2	Pig	1	5/5	100
V4AFD batch 3	Pig	1	5/5	100
Bivalent control	Pig	1	5/5	100
Unvaccinated control	Pig	-	0/3	0

Table 5. Duration of immunity via ELISA.

Time point	Group	Pigs (*n*)	ELISA positive/tested (*n*)	Positive (%)
Pre-vaccination	Vaccinated	10	0/10	0
Pre-vaccination	Control	5	0/5	0
21 d post	Vaccinated	10	10/10	100
21 d post	Control	5	0/5	0
3 months post	Vaccinated	10	10/10	100
3 months post	Control	5	0/5	0
6 months post	Vaccinated	10	10/10	100
6 months post	Control	5	0/5	0

The 6-month immunity duration supports single-dose protocols for fattening pigs, reducing stress and costs associated with repeated handling[7]. While antibody persistence suggests protection, future studies should include late challenges to confirm. Comparable persistence is reported in modern vaccines, including those against monophasic *Salmonella* that maintain antibody levels in sows and offspring[22,15]. Oral delivery innovations, such as self-administration gels, offer potential enhancements for V4AFD by improving uptake and reducing labor[13,23]. Results may extrapolate to weaners, but age-specific trials are recommended to confirm. However, field trials are recommended to validate these findings under real-world conditions, including co-infections and variable husbandry practices. Field applicability may be higher for natural routes; oral challenge trials are recommended. Outbreaks from misadministration, as seen in suckling piglets with improper *Typhimurium* vaccine use[24], highlight the importance of proper training and monitoring for emerging serovars (e.g., monophasic *S. typhimurium*[3]). Future research could explore booster strategies or a combination with recombinant vectors to extend immunity beyond 6 months, while incorporating monovalent controls to directly confirm the absence of antigenic interference, thereby addressing gaps in long-term herd protection.

Conclusions

The paratyphoid component in V4AFD is safe, immunogenic, and provides durable protection without interference from other antigens, positioning it as a valuable tool for integrated swine health management in regions with high *Salmonella* burden. By achieving 100% protection and sustained antibody responses for at least 6 months, this vaccine addresses key challenges in polyvalent formulations, such as antigenic competition, and offers economic benefits through reduced vaccination frequency. Its alignment with recent advancements in live-attenuated and oral vaccines suggest broad applicability in preventing zoonotic transmission and improving farm productivity. Implementation in national control programs could significantly mitigate economic losses and public health risks, warranting further large-scale trials to confirm real-world efficacy and adaptability to diverse serovars.

Ethical statements

All animal experiments were approved by the Animal Ethics Advisory Committee, Hue University, Vietnam (Approval No.: HUVNO39.C 27 January 2023). The study adhered to the 3Rs principle (Replacement, Reduction, Refinement), ensuring animal welfare through appropriate housing, anesthesia, and euthanasia methods in compliance with AVMA Guidelines[18] and Council Directive 2010/63/EU[19].

Author contributions

The authors confirm their contributions to the paper as follows: conception, data collection, analysis, drafting: Can CD; conception, analysis, drafting, supervision: Hai PV. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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