Research Article

Experimental Study on the Components in Polyvalent “Ghost” *Salmonella* Vaccine for Veterinary Use

**Daniela Vasileva Pencheva,¹ Elena Ilieva Velichkova,¹
Denis Zdravkov Sandarov,² Adrian Draganov Cardoso,² Maria Hristova Mileva,³
Petia Dinkova Genova-Kalou,⁴ and Rayna Bryaskova⁵**

¹BB-NCIPD, 26 Yanko Sakazov Boulevard, 1504 Sofia, Bulgaria

²Faculty of Biology, Sofia University “St. Kliment Ohridski”, 8 Dragan Tsankov Boulevard, 1164 Sofia, Bulgaria

³Veterinary Clinic “Zoocenter”, 1799 Sofia, Bulgaria

⁴National Centre of Infectious and Parasitic Diseases, 26 Yanko Sakazov Boulevard, 1504 Sofia, Bulgaria

⁵UCTM, 8 “St. Kliment Ohridski” Boulevard, 1756 Sofia, Bulgaria

Correspondence should be addressed to Daniela Vasileva Pencheva; dani_pencheva@abv.bg

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Development of “ghost” *Salmonella* vaccines, inactivated by using a hybrid nanomaterial based on silver nanoparticles (AgNps) stabilized via polyvinyl alcohol (PVA), is an innovative approach in vaccine production. For this purpose, a series of attempts to establish the components of the polyvalent “ghost” *Salmonella* vaccine and the most suitable methods for its preparation were performed. The following strains *S. Enteritidis*, *S. Newport*-Puerto Rico, and *S. Typhimurium* were chosen as appropriate candidates for their incorporation in order to create polyvalent *Salmonella* “ghost” vaccine for veterinary use.

1. Introduction

Obtaining of “ghost” vaccine by inactivating bacteria with hybrid material based on silver nanoparticles stabilized by polyvinyl alcohol (PVA/AgNps) is an innovative new approach to the application of whole cell inactivated vaccines [1]. There are many advantages of using vaccines treated by this way such as keeping the antigenic range and creating complex protective immunity. Annually in many European countries and the United States are reported a large number of cases of *Salmonella* gastroenteritis. Approximately 80 deaths are recorded each year in the UK [2]. There are also known data caused by a significant number of nontyphoidal *Salmonella* systemic and nonenteric forms of human infections. In a study performed for five-year period in Bulgaria it was found that 21% of them are resistant to ampicillin and gentamicin, 17.64% are resistant to tetracycline, 14.28% are resistant to nalidixic acid, and 10% of them are resistant to chloramphenicol [3]. About

half of the *Salmonella* outbreaks are due to contaminated poultry and poultry products. The route to poultry infection is the colonization of the hen house and its pets, such as rodents, insects, and wild birds. *Salmonella* in the feces of laying eggs contaminate surface or penetrate through the cracks of light shells. Concerning hens with ovarian microorganism infection it was established that *S. Enteritidis* can reach the egg by internal vertical transmission via the reproductive tract to the yolk or albumin [4]. Historically *S. Typhimurium* is the most commonly reported serotype. In 2001, the three most common *Salmonella* serotypes (more than 50% of all isolates) were *S. Typhimurium* (22%), *S. Enteritidis* (18%), and *S. Newport* (10%) [5]. *S. Newport* is one of the *Salmonella* serotypes causing diseases in cattle [6]. The emergence of multidrug-resistant *Salmonella* strains raises the question of strengthening the measures related to the prevention and protection at poultry. An inactivated *Salmonella* vaccine is available on the market for the active immunization of chickens, hens, and their parents [7]. It

contains formalin-inactivated cells of *S. Enteritidis* PT4: 1×10^9 cells and *S. Typhimurium* DT104: 1×10^9 cells. This type of inactivated *Salmonella* vaccines cannot offer 100% protection due to the destruction of bacterial cells as a result of treatment with formalin. An alternative to this vaccine could be a vaccine derived from ghost cells resulting from treatment with the hybrid material.

The aim of the present investigation is to establish the components of the polyvalent “ghost” *Salmonella* vaccine with preserved integrity of the cell surface by inactivation of different *Salmonella* strains with AgNps stabilized by PVA.

2. Materials and Methods

PVA/AgNps hybrid materials were prepared by adding a silver salt (AgNO_3), the precursor for silver ions, to the PVA solution thus leading to coordination of silver ions with hydroxyl groups ($-\text{OH}$) from PVA. Boiling the PVA solution at 100°C for 60 min in the presence of AgNO_3 results in the formation of silver nanoparticles stabilized in PVA, which protects the silver nanoparticles from agglomeration and ensures the homogeneous distribution of silver nanoparticles. The formation of silver nanoparticles was proven by UV-Vis spectroscopy and transmission electron microscopy (TEM) [8]. The silver concentration in PVA/AgNps solution was 174 mg/L as determined by ICP analysis.

To determine the Minimal Bactericidal Concentration (MBC) of the synthesized samples, the following control strains from the collection of “Laboratory for Control of In Vitro Diagnostic Medical Devices” by “BB-NCIPD” *S. Typhi* London, *S. Paratyphi* B, *S. Nairobi*, *S. Typhimurium*, 79a *S. Newport*-Puerto Rico, *S. Enteritidis*, and *S. Enteritidis* ATCC 13076 were used. The PVA/AgNps solution (174 mg/L) is diluted initially with injection water at ratio 1:6 thus leading to starting concentration of 29 mg/L. In five sterile tubes, successively falling twofold dilutions starting from a working dilution of PVA/AgNps in a volume of 1 mL with water for injection to a concentration of 0.45 mg/L were performed. To each tube was added a quantity of the bacterial suspension (by validated patented methodology) to provide from 10^5 to 10^6 CFU (Colonies Forming Units) of them. From a suspension the related positive control was seeded by the same amount using surface method on agar plates with Soybean Casein Agar (SCA). Tubes and plates were placed for 24 hours at $32.5 \pm 2.5^\circ\text{C}$. Each tube was plated by agar surface plate method on plate with SCA, which was cultured in a thermostat at $32.5 \pm 2.5^\circ\text{C}$ in order to confirm inactivation of the bacterial suspension.

From working cultures of 4 control *Salmonella* strains, *S. Typhimurium*, *S. Newport*-Puerto Rico, *S. Enteritidis*, and *S. Enteritidis* ATCC 13076, were prepared as antigens for immunization “ghost” *Salmonella* vaccines. To obtain the required bacterial mass of pure culture, the strain was inoculated on plain agar slant layer. A standardized bacterial suspension from each strain was separately treated with the hybrid material PVA/AgNps, added in an amount that is in a silver concentration of 30 mg/L in the final volume of the antigen. Confirmed inactivated bacterial suspension standardized in densitometer to 3 MF was used as an antigen

FIGURE 1: MBC of PVA/AgNps determined by macrodilution method for (a) *S. Newport*-Puerto Rico, (b) *S. Enteritidis* ATCC 13076, (c) *S. Enteritidis*, and (d) *S. Typhimurium*.

for immunization of rabbits. Intravenous immunizations were carried out with increasing antigenic load of 0.5 to 2 mL as established in the “BB-NCIPD” scheme on Californian rabbits: immunization in vena marginalis in intervals of 3 to 4 days.

The resulting serum was titrated in reaction stage agglutination to establish the specific titer. The presence of cross-agglutinines in different hyperimmune sera was established by slide agglutination reaction (Table 1).

Cell viability after the cytotoxicity analysis of the material was determined by a modification of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] [9] analysis on the cell line of mouse fibroblast cells (L20B). It gives information about the possibilities of application of the hybrid material that will perform bactericidal effect without affecting the metabolism of host cells.

TABLE 1: Content of cross-agglutinins diluted to 1:50 anti-*Salmonella* sera.

Strains	<i>S. enterica</i> serovar Enteritidis ATCC 13076 1,9,12; gm;—	<i>S. enterica</i> serovar Enteritidis 1,9,12; gm;—	79 a <i>S. enterica</i> serovar Newport-Puerto Rico 6,8 [20];—;1,2	<i>S. enterica</i> serovar Typhimurium 1,4,[5],12;i;1,5
Anti- <i>S. Enteritidis</i> ATCC 13076 serum	++++	+	—	++
Anti- <i>S. Enteritidis</i> serum	—	++++	—	+++
Anti- <i>S. Newport-Puerto Rico</i> serum	—	—	++++	++
Anti- <i>S. Typhimurium</i> serum	—	—	+++	++++

++++: very good visible agglutinates in clear liquid; +++: good visible agglutinates in almost clear liquid; ++: visible agglutinates in turbid liquid; +: slightly visible agglutinates in turbid liquid.

3. Results and Discussion

Initially, the Minimal Bactericidal Concentration (MBC) of PVA/AgNps hybrid materials for the organisms *Salmonella enterica* serovar Typhi London, *Salmonella enterica* serovar Paratyphi B, and *Salmonella enterica* serovar Nairobi, which were added to the reaction mixture at a concentration of 10^5 to 10^6 CFU via a validated methodology, was determined according to the requirements of CLSI M 26-A [10]. It was established that the MBC was defined at 0.054 mg/L silver concentration in all cases.

MBC for the strains provided for involvement in the experimental “ghost” *Salmonella* vaccine, *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Newport-Puerto Rico, *Salmonella enterica* serovar Enteritidis, and *Salmonella enterica* serovar Enteritidis ATCC 13076, was additionally determined. Before performing the test, the MBC for both strains *S. enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium was established as lower than 0.027 mg/L. Only for *S. Newport-Puerto Rico* was the MBC 0.108 mg/L (≈ 0.11 mg/L) (Figure 1). Therefore, it was assumed that the tested *Salmonella* strains were sensitive to silver, as tests with the same hybrid material showed that MBC values equal to or more than 1.1 mg/L are sign for silver resistance [11]. Evidence of widespread resistance of *Salmonella* to silver has long been cited in the literature, resulting in a plasmid encoding the genes for resistance to heavy metals [12].

Maximal nontoxic concentration (MNC) was defined as 0.007 mg/L, while the concentration required to inhibit cell viability by 50% (CD_{50}) was determined as 0.53 mg/L in a dose-dependent manner (Figure 2). As the MBC from the respective strains was determined at 10^5 to 10^6 CFU bacterial load, therefore, to inactivate one billionth bacterial suspension, silver concentration of 30 mg/L suspension was applied.

Sera were tested in the reaction slide agglutination at a dilution with TRIS saline buffer as 1:50 for presence of cross-agglutinins from other strains used in the experiment (Table 1). With reference to the scheme of White-Kaufmann [13] common H1 antigens in the second phase of *S. enterica* serovar Newport-Puerto Rico and *S. enterica* serovar Typhimurium were found. This explains the coagglutination in anti-*S. Newport-Puerto Rico* and anti-*S. Typhimurium* sera. Having common O1 antigens explains coagglutination in sera: anti-*S. Enteritidis* and anti-*S. Typhimurium* serum.

FIGURE 2: Cytotoxic effect of PVA/AgNps on the viability of mouse fibroblast (L20B) cell line at 24 h and 48 h.

The specific titer of all obtained after immunization rabbit antisera was determined in a Gruber's reaction stage agglutination. Anti-*S. Enteritidis* ATCC 13076, anti-*S. Newport-Puerto Rico*, and anti-*S. Typhimurium* sera were with O-titer 1:6400. Only anti-*S. Enteritidis* serum has O-titer 1:1600. A significant difference in the activity of sera obtained from both strains *S. Enteritidis* was found; therefore it was considered as appropriate to incorporate both of them in the ongoing prospective studies on the composition of polyvalent *Salmonella* “ghost” vaccine for veterinary use.

TEM analysis a month after completion of the immunization was performed (Figure 3) to one of those used in attempts antigens. It was found that the presence of the PVA/AgNps for longer period in the antigen for the immunization results in complete lysis of the bacterial cells after apoptosis. Therefore, an additional step consisting in washing of the antigen with saline after inactivation with PVA/AgNps, in order to preserve the inactivated bacterial cells in the form of “ghost” cells, is necessary.

4. Conclusions

PVA/AgNps hybrid material was applied to obtain “ghost” cells with preserved integrity of the cell surface

FIGURE 3: TEM analysis of the *Salmonella* antigen, inactivated with PVA/AgNp.

by inactivation of different *Salmonella* strains. Initially, MBC for different *Salmonella* strains was determined by macrodilution method. Minimal nontoxic concentration of PVA/AgNp and CD_{50} were established as well. The specific titer of all obtained after immunization rabbit antisera was determined in a Gruber's reaction stage agglutination, as strains *S. Enteritidis*, *S. Newport*-Puerto Rico, and *S. Typhimurium* were chosen as appropriate candidates for their incorporation in order to create polyvalent *Salmonella* "ghost" vaccine for veterinary use.

The addition of more strains to the vaccine will expand its range of possible causes, and their inactivation by PVA/AgNps will allow retention of a full range of antigenic determinants thus providing complete protection.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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