"BUL BIO-NCIPD" COMPANY WITH TRADITIONS IN VACCINE PRODUCTION AND WITH LOOK AHEAD TO FUTURE

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I. TRADITIONS http://bulbio.com

- **BB-NCIPD** Ltd. - commercial company, 100% state-owned, belongs to the Ministry of Health of Bulgaria, with over 130 years history.
- became a separate entity at the end of 2000 based on the production department of the National Center of Infectious and Parasitic Diseases that had a long history in the manufacture of biopreparations (see Historical notes).
- The production nomenclature covers more than 600 medicines, divided in two main groups:
  - human medicines
  - in-vitro diagnostic medicine products;
- have been implemented new technologies meeting the highest requirements of the international standards;

The vaccines of "**BB-NCIPD**" comply with the **WHO** and **European Pharmacopoeia** requirements

The bio-products of **BB-NCIPD Ltd**. are exported in over 140 countries in the world and this export forms more than 40% of its revenues.

- The production of drugs for human medicine meet the **Good Manufacturing Practice** requirements. **BB-NCIPD Ltd**. holds **production license** (No. I-65/12.02.2003), issued by the **Bulgarian Drug Agency**, which approves it as a manufacturer, who meets the requirements of Human Medicines and Pharmacies Act.
- A **system of quality control** meets the requirements of **ISO 9001:2008** (Certificate **Lloyd's Register** QA No. 368090).

VACCINES

- **Combating vaccine-preventable diseases is a major concern of the health care system** in the advanced countries. The horizontal transmission of infection (from person to person) is difficult or even becomes impossible by preventing replication of the infectious agent through immunization.
- The effects of **reduction of immunization** coverage can be **dangerous and even tragic**.
- **The immunization is widely recognized as the most successful and cost-effective health interventions** ever implemented in public health practice. It prevents between 2 and 3 million deaths each year (http://www.who.int/campaigns/immunization-week/2014/event/en/).

A major producer of vaccines for mass application in Bulgaria is "**BB-NCIPD**."
Nowadays the vaccine-production of "**BB-NCIPD**" is concentrated in the area of bacterial vaccines that according their mechanism of action protect by the following diseases:

- With mucosal replication - pertussis;
- Production of toxins - diphtheria, tetanus;
- Replication in macrophages-TB.

The **improved from the WHO**, vaccines give the ability for its distribution worldwide. Produced are **for the domestic and foreign markets** (*Table 1*) **diphtheria and tetanus toxoid** alone or in combination with **whole cell pertussis vaccine** and the oldest historically among the vaccines, the **BCG vaccine**.
The vaccines are available to our clients **with and without a preservative thiomersal** in **different cuts** and also **in Bulk** as active substances.

	BCG vaccine, freeze-dried (live)	**TETATOX** tetanus vaccine (adsorbed)	**DIFTET** diphtheria and tetanus vaccine (adsorbed)	**DIFTETKOK** diphtheria, tetanus and pertussis vaccine (adsorbed)	**TETADIF** tetanus and diphtheria vaccine (adsorbed)	**ANTI-CHF VACCINE**(inactivated)
Type	BCG - Freeze-dried product	Suspension for injection	Suspension for injection	Suspension for injection	Suspension for injection	Solution for injection
Sizes	Boxes of 20 ampoules each containing 10 doses (plus diluent) Boxes of 20 ampoules each containing 20 doses (plus diluent)	1 ampoule of 1 dose of vaccine 1 vial of 10 doses of vaccine 1 vial of 20 doses of vaccine	1 ampoule of 1 dose of vaccine 1 vial of 10 doses of vaccine 1 vial of 20 doses of vaccine	1 ampoule of 1 dose of vaccine 1 vial of 10 doses of vaccine 1 vial of 20 doses of vaccine	1 ampoule of 1 dose of vaccine 1 vial of 10 doses of vaccine 1 vial of 20 doses of vaccine	50 ampoules of 1 ml of vaccine
Dose	For infants - 0.05 ml I/D Dose Above 1 year of age - 0.1 ml I/D Dose	0.5 ml	0.5 ml	0.5 ml	0.5 ml	1 ml
Contents	Live bacteria derived from a culture of the Bacillus of Calmette and Guerin (BCG), which contains dried suspension of live attenuated strain Micobacterium bovis (Sofia SL222)	Human vaccinating dose 0.5 ml contains: Purified Tetanus Toxoid -not less than 40 IU Aluminium hydroxide (Al +++)-not more than 1.25 mg Thiomersal-not more than 0.05 mg Sodium chloride-not more than 5.00 mg Water for injection-q. s. 0.5 ml	Human vaccinating dose 0.5 ml contains: Purified Diphtheria Toxoid-not less than 30 IU Purified Tetanus Toxoid-not less tnan 40 IU Aluminium hydroxide (Al+++)-not more than 1.25mg Thiomersal-not more than 0.05 mg Sodium chloride-not more than 5.00 mg Water for injection-q. s. 0.5ml	Human vaccinating dose 0.5 ml contains: Purified Diphtheria Toxoid-not less than 30 IU Purified Tetanus Toxoid-not less than 40 IU Inactivated *B. pertussis* suspension -not less than 4 IU Aluminium hydroxide (Al+++) - not more than 1.25 mg Thiomersal -not more than 0.05 mg Sodium chloride -not more than 5.00 mg Water for injection -q.s. 0.5 ml	Human vaccinating dose 0.5 ml contains: Purified Tetanus Toxoid-not less than 40 IU Purified Diphtheria Toxoid-not less than 4 IU Aluminium hydroxide (Al+++)-not more than 1.25 mg Thiomersal-not more than 0.05 mg Sodium chloride-not more than 5.00 mg Water for injection-q.s. 0.5 ml	CCHF antigen - brain suspension of newborn white mice - inactivated
Indications	For the primary immunization of infants and immunization or reimmunization of children and adults who have reacted negatively to the usual tuberculin tests	Specific prophylaxis of tetanus	Combined protection against diphtheria and tetanus.	Combined protection against diphtheria, tetanus and pertussis.	Combined protection against tetanus and diphtheria. for chidren over 7 years of age and adults	Prophylaxis of CCHF
Administr	Intradermaly	Intramuscularly	Subcutaneously or intramuscularly.	Subcutaneously.	Intramuscularly.	Subcutaneously
Storage	Between +2°C and +8°C. Protect from light.	Between +2°C and +8°C in a dark place. Do not freeze.	Between +2°C and +8°C. Do not freeze.	Between +2°C and +8°C. Do not freeze.	Between +2°C and +8°C. Do not freeze!	Between +2°C and +8°C. Protect from light.
Usage	Tuberculin syringe and Mantoux-type needle are used for intradermal application. Special care should be taken to avoid subcutaneous injection. Any open ampoules remaining should be discarded.	Shake before use. Do not use a product that has been frozen.	Shake before use to obtain a homogenous suspension Do not use a product that has been frozen.	Shake before use!. Do not use a product that has been frozen.	Shake before use! Do not use a product that has been frozen.	Opened ampoule should be used immediately.
Exp.	24 months	36 months	36 months	30 months	36 months	24 months

The immunization (reimmunization) with **DIFTET, TETADIF, DIFTETKOK** can be made simultaneously **with other vaccines** such **as poliomyelitis, influenza, hepatitis B, measles, rubella** and **BCG**.

They can be associated **also with immune globulins**. The usage of different syringes, needles and injection side is needed.

When there are **contraindications to pertussis component**, **diphtheria and tetanus vaccine** is applied by the immunization schedule of DIFTETKOK vaccine.

For the needs of **WHO, UNICEF and PAHO** (Pan American Health Organization) are provided bacterial vaccines via our long-standing business partner **Inter Vax, Canada**.

Produced is also **PPD Tuberculin**, ready to use for Mantoux's intradermal test to assist in clinical diagnosis of tuberculosis.

The only **virus vaccine** that is produced in the company is inactivated **vaccine against Crimean Hemorrhagic Fever** (**CHF**). It contains **inactivated virus** as antigen strain of CHF V 42/81 and administered prophylactically population in endemic distribution of the causative regions. Two applications of the vaccine provide specific immunity and prevent disease CHF.

We are currently running a **joint project** to **develop a new recombinant DNA vaccine against CHF** in partnership with Canada and Kazakhstan.

II. FUTURE DEVELOPMENTS

- Apart from improving animal health and productivity, veterinary vaccines have a **significant impact on public health** through reductions in the use of veterinary pharmaceuticals and hormones and their residues in the human food chain.
- According to the **European Pharmacopoeia 8.0.** Chapter "Vaccines for veterinary use", **the bacterial strain is permitted to be modified by genetic engineering**, such as the identity, purity and antigen activity of each bacterial culture used must be carefully controlled.
- A new approach in the **design of recombinant vaccines** are inactivated vaccines containing whole cells. Successfully manipulation of the bacterial genome could provide **surface-presented antigens of various pathogens**. The challenge here would be the use of **innovative methods for inactivation** of bacterial vaccines.

Treating the development vaccine with a **hybrid material containing silver nanoparticles** will inactivate the strain and as a result can be obtained recombinant “ghost” cells.

“Ghost” vaccines are an innovative idea to obtain better results of immunization due to the presence of fuller spectrum of saved antigenic determinants and development of protective immunity.

Development of a **vaccine for veterinary use of recombinant "ghost" cell carriers of the bacterial genomes of different pathogens** against causes of enteric disease is the basis of a draft proposal with potential awaiting development and implementation.

To achieve this main goal we should go a long way of experimental research.

The choice of the **components of a polyvalent vaccine against enteric diseases in animals** is a first important step in its development.

It is known that a **traditional production of non-living** (killed) **vaccine by heat treatment, irradiation or chemical treatment** of the pathogen often leads to **denaturation of significant structural components of the cell wall**, changing the antigenic character of the vaccine and due to the loss of important immunogenic epitopes cannot create a complete immunity

Obtaining of "ghost" vaccine by **inactivating bacteria with hybrid material** based on silver nanoparticles stabilized by polyvinyl alcohol (PVA/AgNps) and **keeping the antigenic range and creating of complex protective immunity** is an innovative new approach to the application of whole cell inactivated vaccines.

Experimental study on the components in poly-valent "ghost" Salmonella vaccine for veterinary use

- 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.
- There are also known data caused by a **significant number of non-typhoidal Salmonella systemic and non-enteric forms of human infections**.
- In a study performed for 5 year period in Bulgaria it was found that **21% of them are resistant to A and G, 17.64% are resistant to T, 14.28% to Nx and 10% -resistant to C.**
- The emergence of **multidrug-resistant Salmonella** strains raises the question of **strengthening the measures** related to the prevention and protection at poultry.

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 penetrated** through the cracks of light shells.

At **hens with ovarian infection** was established that ***S. Enteritidis*** can reach the egg by **internal vertical transmission** via the reproductive tract to the yolk or albumin.

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%),** ***S. Newport*** **(10%).**

S. Newport is one of the Salmonella serotypes causing diseases **in cattle**.

Alternative to the available at the market inactivated with formaldehide Salmonella vaccines could be a **vaccine derived from ghost cells** resulting from treatment with the hybrid material PVA/AgNps.

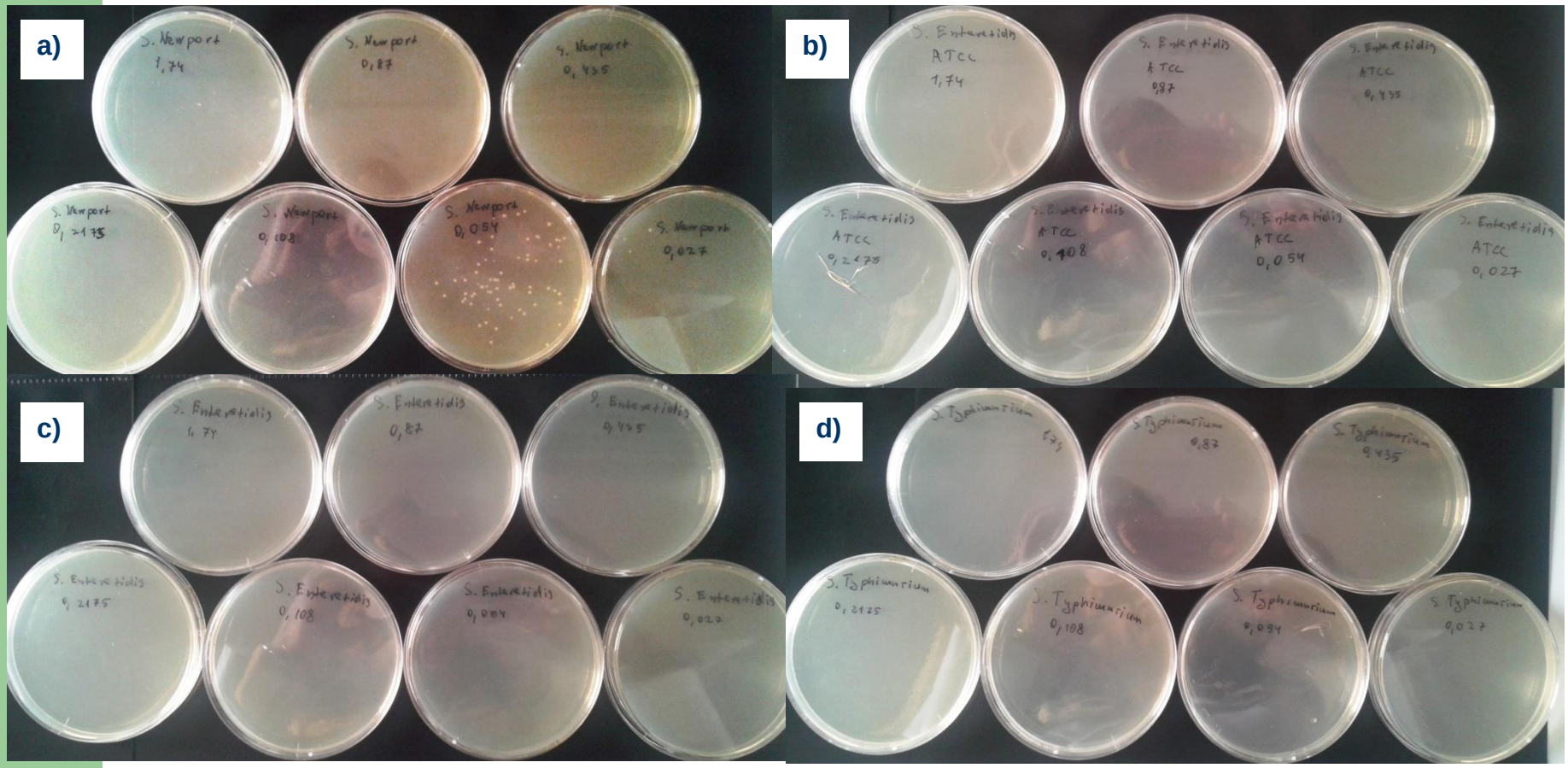
The aim of the first investigation was to establish the components of the **poly-valent "ghost" Salmonella vaccine** by inactivation of different Salmonella strains - two strains ***S.Enteritidis, S. Newport Puerto Rico*** and ***S.Typhymurium***.

Initially, **MBC** for different Salmonella strains was determined by macrodilution method (Figure 1).

The MBC for both strains ***S. enterica* serovar Enteritidis and *S. enterica* serovar Thyphimurium** was established as lower than 0.027 mg/L. Only for ***S.Newport- Puerto Rico*** the MBC was - 0.108 mg/L (≈0.11 mg/L).

T**he tested Salmonella strains were sensitive to silver**, as tests with the same hybrid material showed that MBC values **equal or more than 1.1 mg/L are sign for silver resistance.**

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.*



The **Maximal non-toxic concentration** (MNC) is the maximal concentration, that altered neither the morphology of monolayer nor the cell survival rate. **MNC was defined as 0.007** mg/L.The concentration required to inhibit cell viability by 50% (**CD50**) was determined as **0.53 mg/L** in a dose-dependent manner(Figure2).

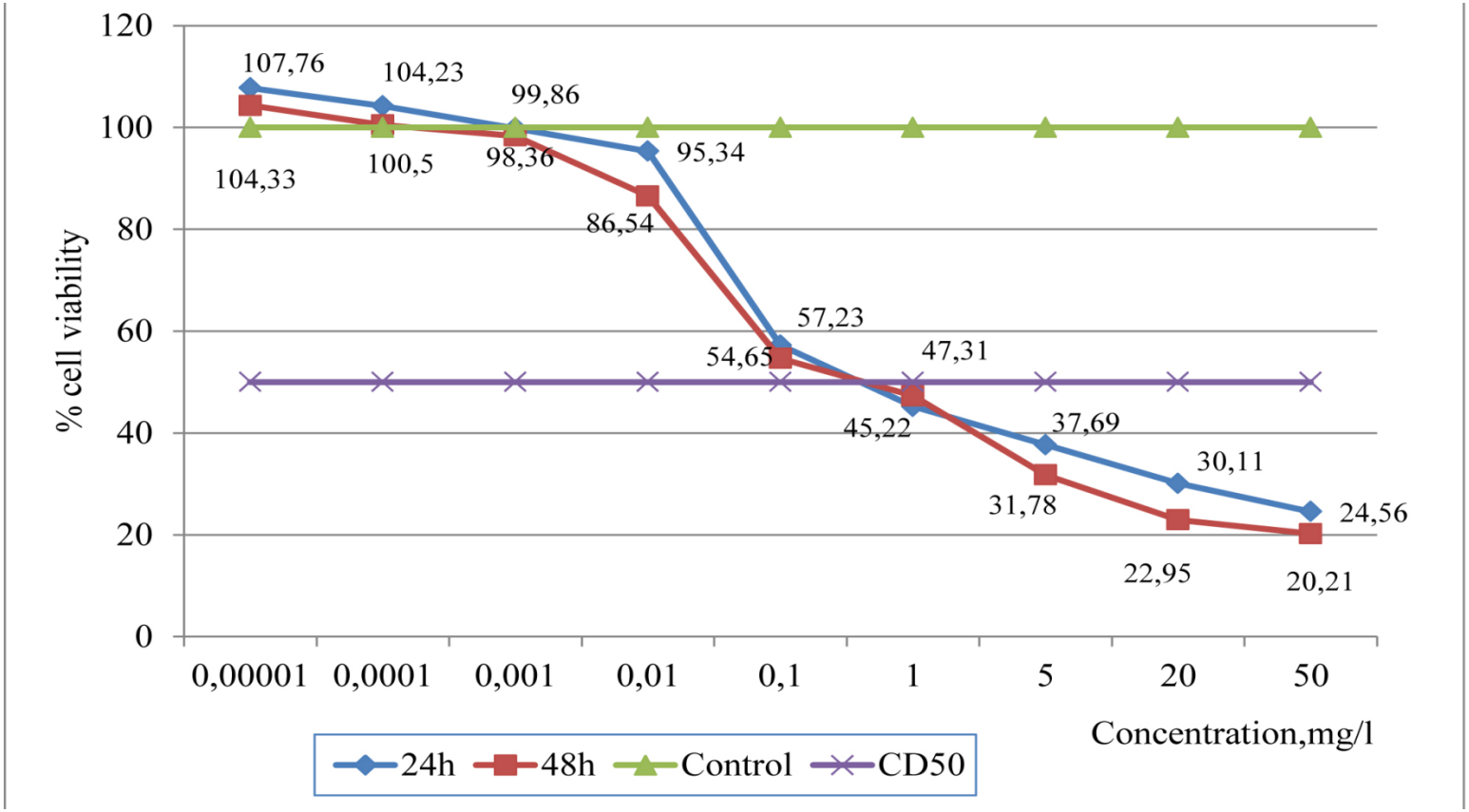


Figure 2. Cytotoxic effect of PVA/AgNps on the viability of **mouse fibroblast (L20B) cell line** at 24h and 48h

As the MBC from the respective strains was determined at 10^5 - 10^6 CFU bacterial load, therefore **to inactivate one billionth bacterial**, silver concentration **of 30 mg /L** suspension was applied.

From working cultures of the 4 control Salmonella strains – ***S. Typhimurium*, *S. Newport- Puerto Rico*, *S. Enteritidis*, *S. Enteritidis ATCC* 13076**, were prepared as antigens for immunization "ghost" Salmonella vaccines.

The inactivation of the bacteria was confirmed with cultural method.

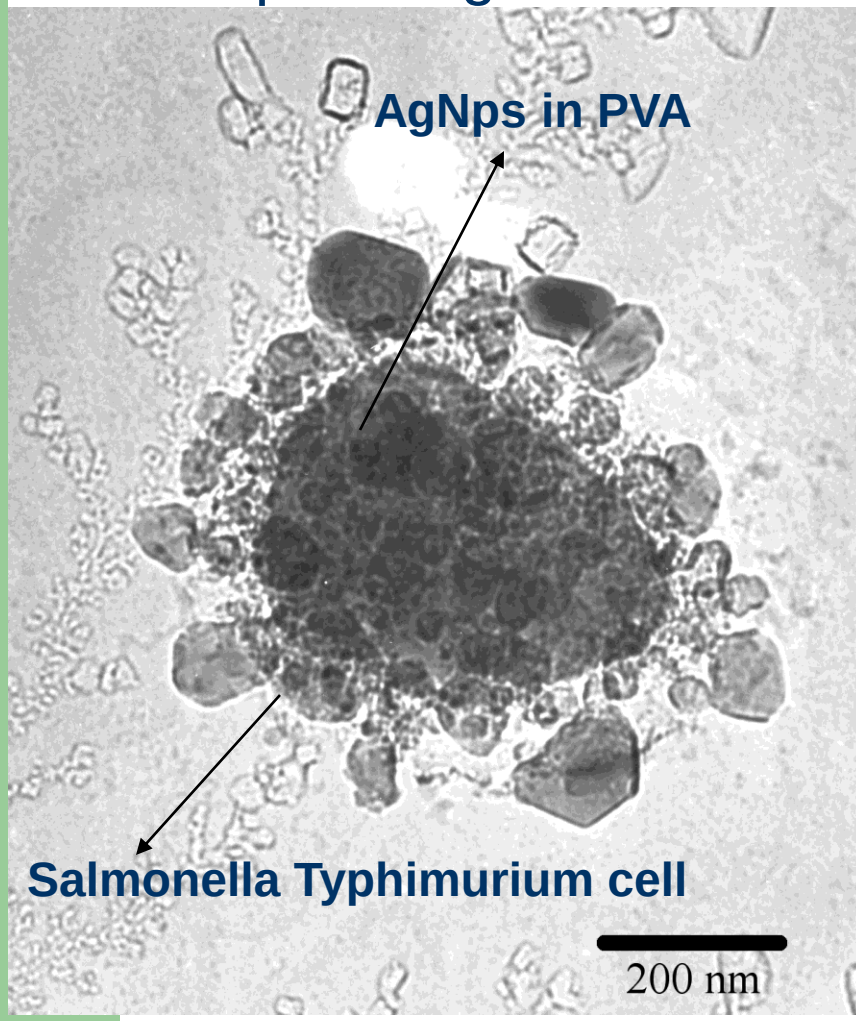
Bacterial suspension was **standardized in densitometer to 3MF** and used as an antigen for intravenous immunization of Californian rabbits with increasing antigenic load of 0.5 to 2 ml by established in the "**BB-NCIPD**" scheme - in vena marginalis in intervals of 3 to 4 days .

The **specific titer of** all obtained after immunization rabbit **Salmonella antisera** was determined in a Gruber's reaction stage agglutination. The antisera were
with O-titer 1:6400 with exception of the anti- *S. Enteritidis* serum, that has O titer 1:1600.
It was found a significant **difference in the activity of sera, obtained from both strains *S. Enteritidis*** (Table 2), therefore it was considered **to incorporate both of them** in the polyvalent Salmonella "ghost" vaccine for veterinary use.

	***S. enterica* serovar Enteritidis ATCC13076 1,9,12;gm;-**	***S. enterica* serovar Enteritidis 1,9,12;gm;-**	***79 a S. enterica* serovar Newport Puerto Rico 6,8 [20];-;1,2**	***S. enterica* serovar Typhimurium 1,4,[5],12;i;1,5**
Anti- *S. Enteritidis* ATCC13076 serum	++++	+	-	++
Anti- *S. Enteritidis* serum	-	++++	-	+++
Anti- *S. Newport* Puerto Rico serum	-	-	++++	++
Anti- *S.Typhimurium* serum	-	-	+++	++++

Legend: ++++ 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.

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 after inactivation with PVA/AgNps,** in order to preserve the inactivated bacterial cells in the form of "ghost" cells is necessary.

Experimental research of polyvalent “Ghost” *Escherichia coli* vaccine

1. ***E.coli* O104**

- The outbreak from ***E.coli* O104:H4** in Germany and other EU/EEA countries was one of the largest reported **HUS** (Hemolytic – uremic syndrome) **outbreaks** in the world.
- **The enterroaggregative Verotoxin** (Vtx-) producing *E.coli* strain (EAggEC)/VTEC) serotype O104:H4 has often been described as an enterrohaemorrhagic *E.coli* (EHEC).
- VTEC that produce Attaching and Effacing (AE) lesions on enterocytes are **EHEC.**

Interesting fact is that **the primary sources and vehicles of** typical **EHEC infections in humans are ruminants**, whereas **no animal reservoir has been identified for enteroaggregative *E.coli***.

The VTEC sero-group O104 has been reported three times as **isolate from animals and food** by the EU member states:

- Two of the isolations were **from cattle** and the detected **serotypes** were O104:**H12** and O 104:**H21**.
- VTEC serotype O104 was isolated also **from wild boar**.
- From **sheep and young cattle** was isolated O104:**H7**.
- In food VTEC O104 was isolated **from bovine carcasses and meat**.

When **infecting humans VTEC** can also be responsible for **HUS** due to the production of Vtx.

Serotype *E.coli* O104:**H21** was also agent of **sporadically outbreaks**.

Although **the main agent of two HUS cases** in German was *E.coli* O104:**H4** VTEC strain.

The strategy of the present study was to **create "ghost" *E.coli* O104 cells** using the hybrid material, synthesized according to reported in the literature method (Figure4).

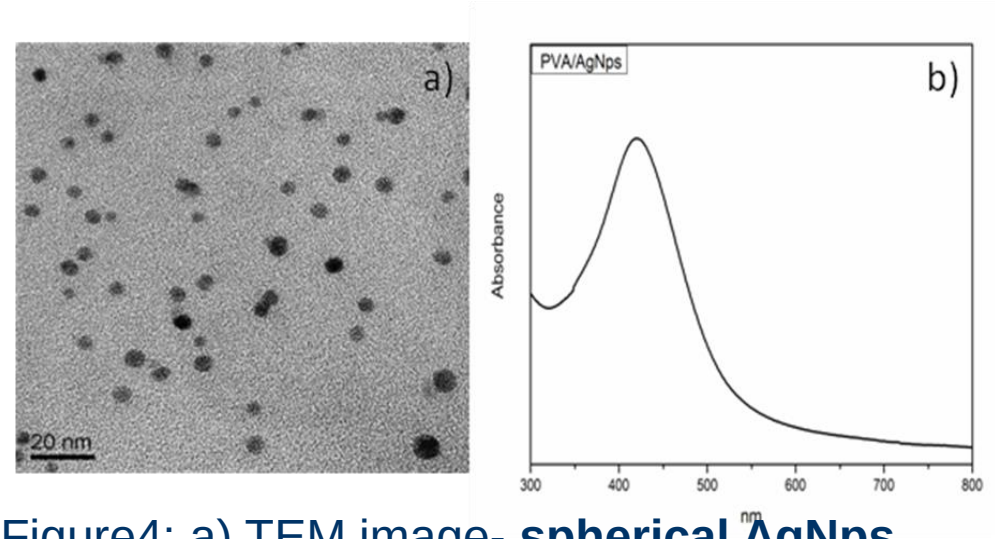


Figure4: a) TEM image- **spherical AgNps** with an average **diameter of 5.0 ± 1.0 nm.**

b) UV-Vis spectroscopy confirmed the presence of AgNps by appearance of strong **absorption bands at 420 nm**.

The determined **MBC**s of *E. coli* O104 was **0.054** mg/L, which demonstrated **sensitivity to silver**.

For **the inactivation** process, PVA/AgNps solution with silver concentration of **30 mg/L** was used.

The process of inactivation was confirmed onto cultural method.

The value of **therapeutic efficacy** (TE) was **75.71**.

MBC and the evidences of TE can determine the **secure intravenous administration of the vaccine suspension.**

The excess of the hybrid material, used for inactivation in **concentration (30 mg/L),** was **removed due washing partically in advance**.

Two rabbits that were put into immunization scheme were elected **from one litter** in order to provide closely related signs and immunity. They passed the full course of **4 immunizations** with increasing antigenic load of **“ghost”** ***E. coli*** **O 104**.

- One of rabbits was immunized with the antigen **inactivated by the hybrid material** (rabbit No1).
- The other rabbit was **immunized with antigen** prepared in a conventional manner – **treated with heat** (rabbit No2)**.**

It was established that the rabbit immunized with **“ghost” bacterial cells forms more rapidly titre of specific antibodies** from those that has been immunized with the antigen treated by the classical method (**Table 2)**.

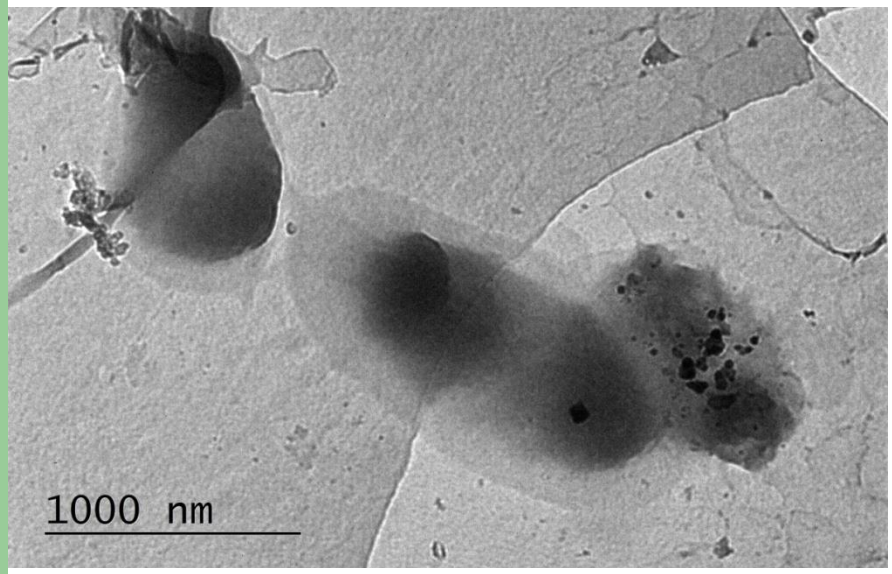
The presence of a **specific titer after the second immunization** was observed only by rabbit No1.

Table2: Determination of specified antibodies against *E.coli* O 104 during and after the end of immunization scheme.

	Titer 3 days after the first immunization	**Titer 3 days after the second immunization**	**Titer 3 days after the third immunization**	**Titer 3 days after the fourth immunization**
Rabbit No 1	Without titer	At **1:50** dilution good positive slide agglutination	At **1:400** dilution good positive slide agglutination	At **1:400** dilution very good positive slide agglutination
Rabbit No 2	Without titer	Without titer	At **1:50** dilution very good positive slide agglutination	At **1:400** dilution very good positive slide agglutination

Legend: Rabbit No1 – immunized with antigen treated with hybrid material; Rabbit No 2 – immunized with antigen treated with heat.

TEM image (Figure5) demonstrates **the changes in the cell structure** of the received at this manner ***E.coli* O104 "ghosts"**.



The picture shows the **advantage in the introduction of washing step after the inactivation** of the antigen with the polymer in a method of treatment.

Figure 5: TEM image of the *E.coli* O 104 – "ghost" cells with discarded cellular content and visible presence in a cell of PVA/AgNps.

I**nfection per os of the immunized rabbits** was provided with **1 ml** of a **billionth suspension of alive bacterial cells *E. coli* O 104.**

The rabbits showed a **mild discomfort during the next day** with transient **loss of appetite**. After this period they **recovered without clinical signs of disease**.

2. ENTEROTOXIGENIC E.coli (ETEC)

ETEC produce one or more **fimbrial adhesins** that mediate **their attachment to specific receptors on mucosal epithelial cells**, producing of **enterotoxines.** This change the water and electrolyte efflux of the small intestine **and lead to neonatal diarrhea and post-weaning diarrhea in farm animals**.
For **protection against ETEC diarrhea** are commonly used **commercially available vaccines,** that are given parentally. They content inactivated **whole-cells**, **purified fimbrial subunit or heat labile enterotoxin (LT).**
Fimbrial adhesins of neonatal porcine **ETEC** are **F4** (K88), **F5** (K99), **F6** (987P) and **F41**.
The **most responsible for diarrhea in young pigs are the F4 ETEC strains** and **for post weaning diarrhea – F4 or F18**.
Some F18 ETEC strains also **produce Shiga Like Toxin IIe (SLT IIe)** and can **cause oedema disease** and not diarrhea.

Verotoxigenic *E.coli* (VTEC) on animals and food were monitored and the report covers primarily **VTEC O157 H7** on the **skin of young cattle and sheep fleeces**.
The monitoring is extended to the ***E.coli* serogroups O26, O103, O111 and O145**, which also cause human infection.

In the last provided (still unpublished) experiment immunization was conducted according to the established schedule of **two rabbits**.

For the test was chosen the strain ***Escherichia coli* O 157 H7**.

The **MBC** of PVA/AgNps was established in this case at silver concentration **0,03 mg/L** and show sensitivity to silver.

Both antigens of *Escherichia coli* **O 157 H7** were prepared as inactivated in **two different ways**:

- the first - **with the hybrid material;**
- the second **- with formalin**.

The **formalin,** added for the inactivation of the second immunization antigen E.coli O157H7, was **in quantities equal to the volume of the added polymer** to the first antigen.

The suspensions are **washed aseptic twice with injection water after centrifugation** of 5000-6000 rpm for 15 minutes to remove the added inactivators.

TEM image of the treated with PVA/AgNps antigens for immunization shows **the existence of ghosts - cells** in the first antigen (Figure 6) with **removed cell content and preserved cell wall**.

The TEM image of cell of the second antigen, treated with formalin (Figure7) shows presence of a **strong thinning of the cell shell**, in some places as **eaten away**.

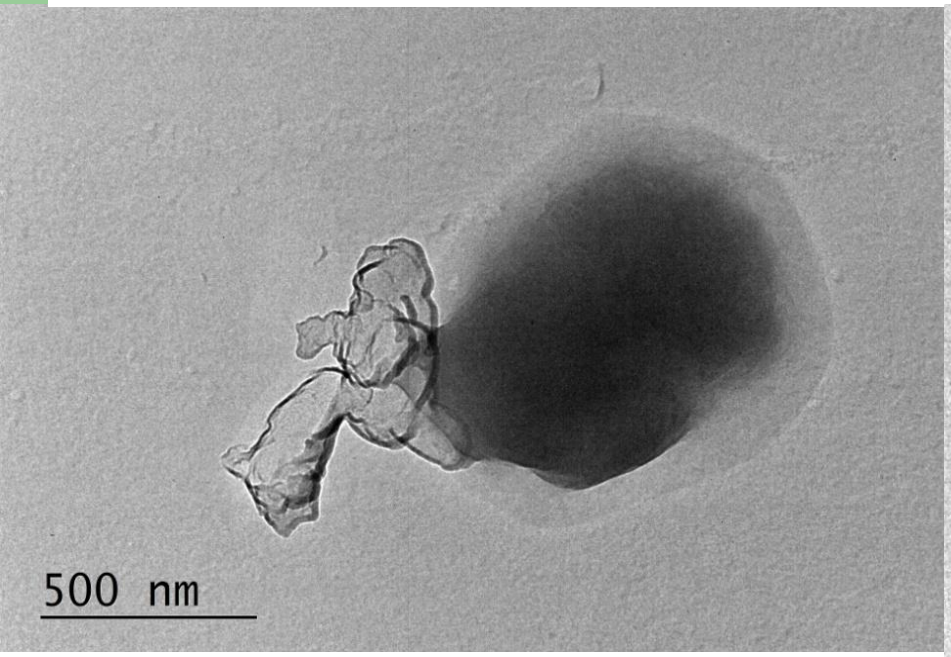


Figure 6: E.coli **O157H7 treated with PVA/AgNps .**

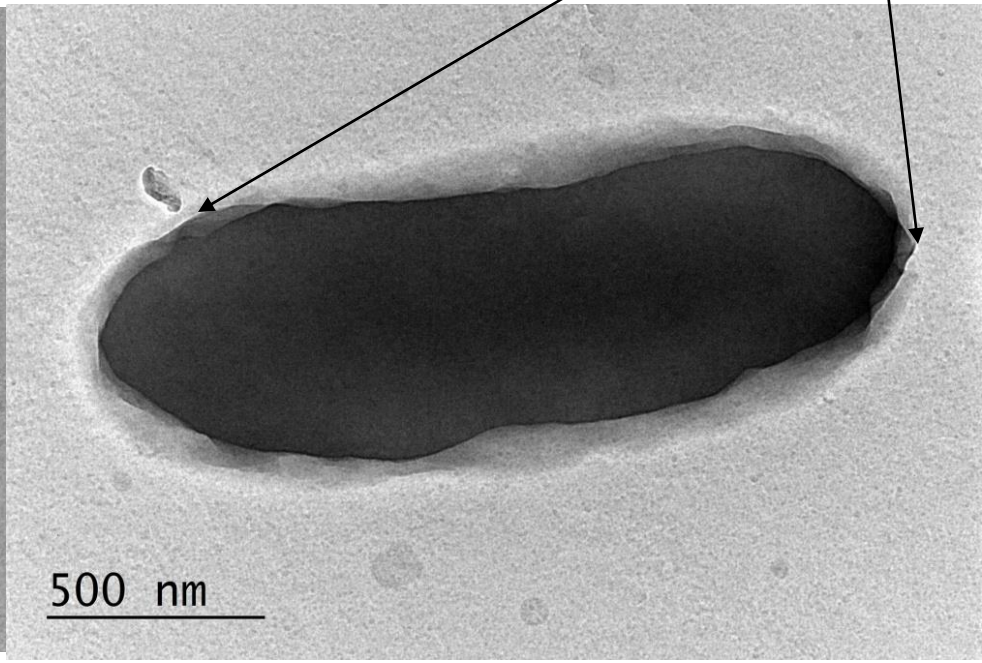


Figure 7: E.coli **O157H7 treated with formaldehyde**.

To establish the influence of the processing of the antigen before each subsequent immunization have been blood samples taken to determine in stage agglutination reaction the reached specific titer (Table 4).

Table 4: The specific titers of sera from the three immunized rabbits after second, third and fourth immunization.

Serum obtained after an immunization with	**Specific titers after the second immunization**		**Specific titers after the third immunization**		**Specific titers after the fourth immunization**	
	O-titer	K-titer	O-titer	K-titer	O-titer	K-titer
E.coli O157H7, treated **with PVA/AgNps**	800	0	1600	200	6400	800
E.coli O157H7, treated **with formaldehide**	200	0	1600	200	6400	400

CONCLUSION

1. The study showed that **both strains *S. Enteritidis, S. Newport-Puerto Rico* and *S. Typhimurium*** are appropriate to be chosen as candidates for their incorporation in order to create “ghost” vaccine for veterinary use.
2. “Ghost” ***E.coli* O104** vaccine **creates protective immunity**.
3. The **TE was established as very good**, which allows the intravenous use of the hybrid material without expecting pathological changes in cells.
4. It was proven **advantage when using antigen, inactivated by the PVA/AgNps hybrid material** (E.coli O104, O157H7) **to such treated by classical methodology** (by heat inactivation or by formaldehyde), expressed in **faster development of specific titer**.

THANK YOU FOR YOUR ATTENTION !

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