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SPECIFIC ASPECTS OF *AVIBACTERIUM PARAGALLINARUM* INACTIVATION WITH FORMALDEHYDE AND THIOMERSAL

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SUMMARY

The paper demonstrates results of testing different modes of *Avibacterium paragallinarum* inactivation with formaldehyde and thiomersal. The bacterium destruction by 0.20% and 0.10% formaldehyde proceeds at the constant rate thus indicating exponential dependence of the microorganism inactivation processes. This fact allows for calculation of the inactivation rate constant that amounts to 2.94 ± 0.37 h^{-1} for 0.10% formaldehyde and 5.86 ± 0.72 h^{-1} for 0.20% formaldehyde. Inactivation using formaldehyde at final concentration of 0.10% at 37 °C and continuous stirring (60 rpm) produces 7.0 dm^3 of bacterin at concentration of 9.5 ± 0.2 lg microbial cells (mc)/cm^3 in 4.3 ± 0.1 h. Thiomersal demonstrated bactericidal action against *Avibacterium paragallinarum* at concentration of 0.04% (1:2500) or higher. Herewith, inactivation process is specified by linearity and the inactivation rate constant amounts to 7.92±1.12 h^{-1}. Under thiomersal sublethal concentration of 0.2% (1:5000) the survival curve is of irregular shape. However, the process of the microorganism death is not exponential, and under continuous decrease, the inactivation rate is going to zero thus making impossible the calculation of the inactivation rate constant. Inactivation mode involving use of 0.04% thiomersal at 37 °C allows production of 7.0 dm^3 of bacterin at 9.5 ± 0.2 lg mc/cm^3 concentration in 5.8 ± 0.1 h. Right after production, the hemagglutination activity of the thiomersal inactivated antigen was higher as compared to formaldehyde inactivated antigen ($P < 0.05$). However, after 10-month storage the activity of the thiomersal inactivated antigen decreased by 3.1 $\log_2$ ($P < 0.05$), and of the formaldehyde inactivated antigen – by 0.4 $\log_2$ ($P > 0.05$). The antigen produced using formaldehyde maintains high hemagglutination activity during storage that is critical for high quality vaccine production.

Key words: infectious coryza (*Haemophilus* infection), strain, inactivation, formaldehyde, thiomersal, antigen, *Avibacterium paragallinarum*.

INTRODUCTION

One of the aspects causing decreased economic performance at poultry farms is respiratory diseases, which may be quite variable in their origin and clinical and morphological manifestation [12].

Out of avian respiratory diseases, infectious coryza (*Haemophilus* infection) is of a particular interest for veterinarians. This is explained on the one hand by scarcity of information and on the other hand by the absence of domestic vaccines for specific prevention [3, 5, 14].

For infectious coryza specific prevention inactivated vaccines are usually used, as they are significantly better than the live ones. First, they are highly safe and innocuous, standardizable, make it possible to inject a dosed amount of the specific antigen; their major biological properties are stable; they induce a strong and long-term immunity and can be used in polyvalent and combined forms [1, 2, 8].

Avibacterium paragallinarum virulent strains are used for the production of inactivated vaccines. The selection of an inactivant and optimal inactivation conditions, which enable to completely deprive the agent of infectivity and maintain antigenicity to the maximum extent, is essential for the preparation of a high quality bacterial antigen. Inactivation process is highly dependent on the performance conditions, purity of bacteria-containing suspension, inactivation agent concentration and type [1, 2, 5, 6]. It is impossible to prepare a high quality antigen with stable immunobiological properties without studying bacteria inactivation processes.

There is no consensus among the researchers involved into development and production of vaccines against

infectious coryza on the effect of different chemical inactivation agents. Some papers demonstrate that thiomersal-inactivated *A. paragallinarum* antigens are more active than formalin-inactivated antigens [7, 8, 11]. Moreover, it is noted that formalin is not an ideal inactivant, because when reacting to amino acid amino groups, it denatures proteins. Formalin-inactivated vaccines based on alumen, aluminum hydroxide gel and mineral oil were less immunogenic than analogous products, inactivated with thiomersal [7, 11]. In practice, 0.01–0.02% thiomersal (0.1–0.2 mg/cm^3) is usually used to preserve immunobiological products. Thiomersal cytotoxic effect is based on interaction and blocking of SH-proteins of cell membrane and enzymes. Surface enzyme inactivation results in bacterial growth inhibition, and intracellular enzyme inactivation leads to bactericidal effect [8, 11].

According to other reports, formalin may be used to prepare bacterial antigens with high immunogenic properties [10, 13]. Predominantly formaldehyde effects polynucleotides and protein amino groups. Formaldehyde reactions to proteins and nucleic acid lead to formation of stable intermolecular cross-links between amino acids and one or two nucleic acid base. This mechanism is accompanied by primary damages, despiralization and inactivation of nucleic acids, significant conformation changes in polypeptide chain and stabilization of biopolymer macrostructure. Besides, formaldehyde damaging effect targets bacteria envelope, cytoplasm membrane as well as intracellular enzymes. 0.1% formalin (by volume) kills vegetative cells of *Pasteurellaceae* bacteria within several hours. If concentrations are lower, it has a bacteriostatic effect, which can be reversible if sodium sulfite is added. At the same time, toxicity is inhibited and biopolymer antigenic and immunogenic properties are stabilized; this property underlies the production of immune-biological products. Formalin products are usually innocuous, highly antigenic, potent and stable if stored for a long time [4, 9, 11].

The purpose of this study was to select an inactivant and optimize inactivation process for *A. paragallinarum* bacteria.

MATERIALS AND METHODS

A. paragallinarum strain No. 5111, serogroup B, deposited in the Collection of strains and microorganisms of the FGBI "ARRIAH" in 2017 was used in the study.

A. paragallinarum was cultured by submerging in 7.0 dm^3 bioreactor Biotron LiFlus GX for 12 hours at 37 °C. Casein soybean digest broth based on enzyme casein hydrolysate and Sigma soybeans with 20 μg/ml NAD (AppliChem) and 5% horse sera ("Microgen") added was used as a nutrient medium.

100 cm^3 of *A. paragallinarum* strain broth culture, collected at the end of the exponential growth phase – beginning of a stationary growth phase at the concentration of 9.5 ± 0.2 lg CFU/cm^3 was transferred to conic vials and placed into Biosan ES20 orbital shaker-incubator. Inactivation was carried out at 37 °C, stirred at 60 rpm.

36% formaldehyde solution (formalin) (Metafrax Company) and thiomersal, containing 46% of mercury (Ferak Berlin, Germany) were used for bacteria inactivation.

The concentration of live microbe cells in suspension were determined by seeding of ten-fold culture dilutions on a dense nutrient medium by a conventional method.

The inactivation parameters were calculated according to the following formula [5]:

$$e^{-kt} = X_i/X_0,$$

where k is an inactivation rate constant (h^{-1});

t is inactivation time (h);

X_0 is an original concentration of microbial cells (lg CFU/cm^3);

X_i is a residual number of microbial cells (lg CFU/ cm^3).

Antigen haemagglutinating activity was measured in haemagglutination assay (HA) using two-fold dilutions (from 1:50 to 1:3200) by a conventional method. The reaction was interpreted visually and scored (crosses). The highest titre dilution resulting in a distinct RBC agglutination was taken as antigen titre (+++ or ++++). At the same time, RBC spontaneous agglutination was tested (negative control). Confidence interval for differences in antigen haemagglutinating activity was 95%.

RESULTS AND DISCUSSION

To optimize *A. paragallinarum* bacteria inactivation using formalin it was necessary to study the survival rate of these microorganisms if exposed to different formalin concentrations.

To study inactivation process formalin was added to broth cultures in the following amounts (0.05%, 0.10% and 0.20% by volume). Live microbial cell concentration was measured before adding inactivant (X_0) and after that and every 60 minutes (X_i) (see Fig. 1).

Figure 1 shows graph lines of *A. paragallinarum* bacteria inactivation when exposed to different concentrations of formalin as well as natural death kinetics (DK).

Data of tests performed suggest that *A. paragallinarum* bacteria when exposed to 0.20 и 0.10% formalin die at a constant rate, this means microorganism inactivation processes are in exponential relationship. This fact enabled to calculate inactivation rate constant (k), which was k = 2.94 ± 0.37 h^{-1} for 0.10% formalin and k = 5.86 ± 0.72 h^{-1} for 0.20% formalin.

The analysis of these k values showed positive correlation between microorganism death rate and inactivant concentration. Moreover, doubling of inactivating agent amount raised k value and reduced inactivation time by a relevant factor. In this case, it may be said that *A. paragallinarum* bacteria inactivation using formalin is a completely predictable process. At the same time bacteria inactivation graph with 0.05% formalin was non-linear.

Thus, being guided by the principle of gentle inactivation and taking into account the fact that lowest possible

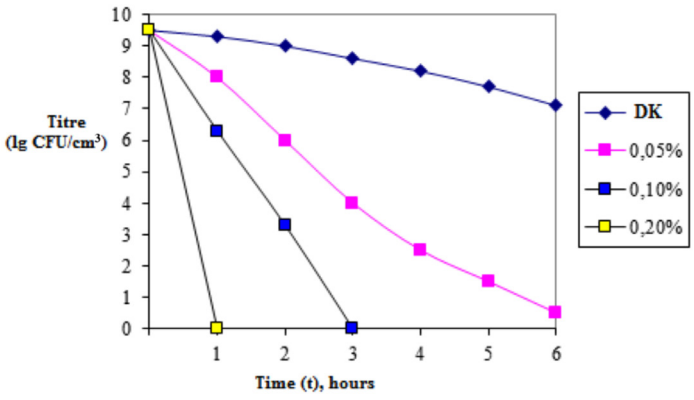

Fig. 1. A. paragallinarum bacteria inactivation kinetics when exposed to formalin, n = 3

formalin concentrations may result in non-linear inactivation processes, we established that 0.10% formalin is the optimal concentration for inactivation of these bacteria.

As there is always a possibility that at least one microorganism can survive in the volume unit of the inactivated suspension the next step was to determine the inactivation time (t).

When the culturing process was over 7.0 l of formalin was added to *A. paragallinarum* No. 5111 broth suspension at the rate of 0.10% by volume.

For t value calculation, the acceptable level of residual virulent material in broth culture depending on its volume was taken into account. As the working volume of inactivated suspension was equal to 7.0 dm^3, the number of survived bacteria could not be higher than 3.84 lg CFU/cm^3.

Figure 2 shows *A. paragallinarum* bacteria inactivation graph when exposed to 0.10% formalin based on inactivation rate constant and acceptable level of residual virulent material.

Calculated inactivation time showed that used formalin amount ensured bacteria death level of 3.84 lg CFU/cm^3 within 4.3 ± 0.1 h.

Thus, optimized *A. paragallinarum* inactivation mode using 0.10% formalin at 37 °C and constant stirring (60 rpm) allows to produce 7.0 l of bacterin at the concentration of 9.5 ± 0.2 lg m.c./cm^3 within 4.3 ± 0.1 h.

The next stage of the study was optimization of *A. paragallinarum* inactivation with thiomersal and its kinetics analysis. For this purpose, bacteria suspension broth, taken at the end of the exponential growth phase – at the beginning of the stationary growth phase, was exposed to different thiomersal dilution concentrations (0.02, 0.04 and 0.08%).

Figure 3 shows bacteria inactivation graphs when exposed to different thiomersal concentrations.

In the course of the tests performed it was established that thiomersal is bactericidal for *A. paragallinarum* cells when its concentration is equal to 0.04% (1:2500) or higher. Herewith the inactivation process was linear, and that enabled to calculate the inactivation rate constant (k = 7.92 ± 1.12 h^{-1}). When exposed to inactivant at sublethal concentration of 0.02% (1:5000), the survival graph line had a complex configuration and consisted of the starting segment showing rapid inactivation and following segment of slow inactivation. At the same time, the microorganism death process was not in exponential relationship, and inactivation rate value was constantly decreasing and approached zero, that is why the k value calculation was not possible.

In further studies 0.04% thiomersal was used for *A. paragallinarum* bacteria inactivation.

At the next stage, the duration of microorganism inactivation in a working volume of 7.0 l was determined. Inactivation was carried out under standard conditions at the temperature of 37 °C and constant stirring at 60 rpm.

Figure 4 shows *A. paragallinarum* bacteria inactivation when exposed to 0.04% thiomersal taking the inactivation rate constant and acceptable level of residual virulent material into account.

Inactivation time calculation findings proved that the use of the said thiomersal concentration ensured bacteria death up to the level of 3.84 lg CFU/cm^3 within 5.8 ± 0.1 h.

Thus, the optimized inactivation mode using 0.04% thiomersal enables to produce 7.0 dm^3 of bacterin at concentration of 9.5 ± 0.2 lg m.c./cm^3 within 5.8 ± 0.1 h.

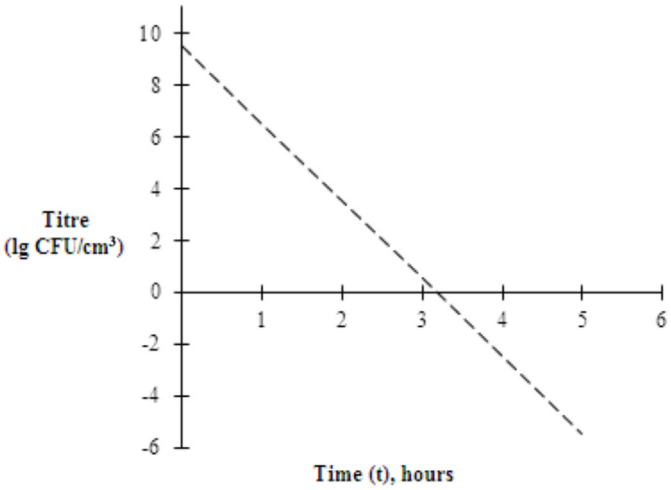

Fig. 2. A. paragallinarum bacteria inactivation with formalin at final concentration of 0.10%, n = 3

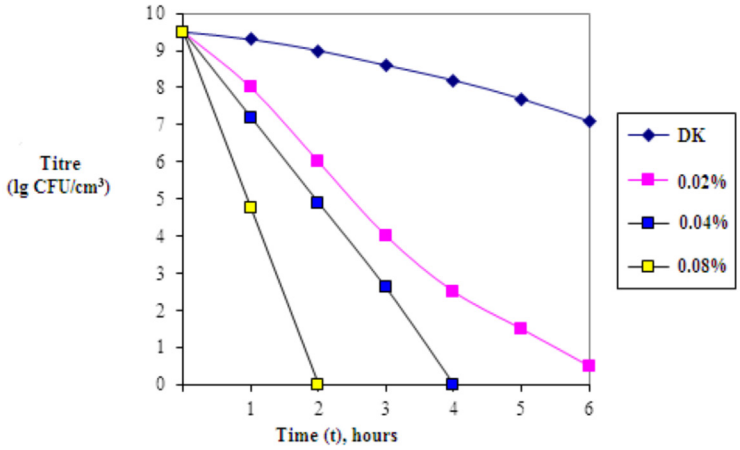

Fig. 3. Kinetics of A. paragallinarum inactivation with thiomersal, n = 3

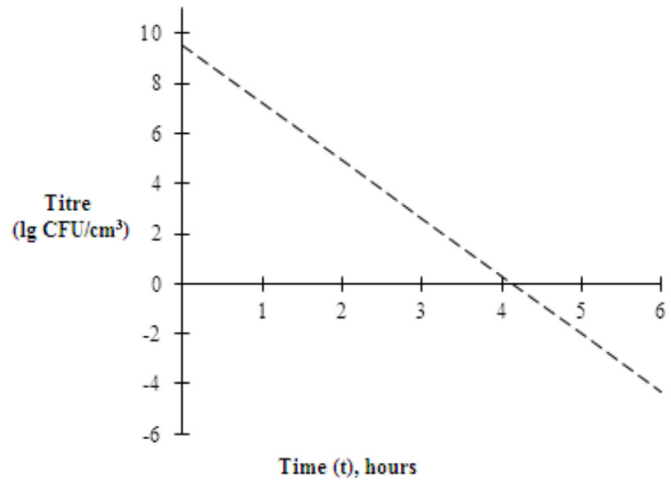

Fig. 4. A. paragallinarum bacteria inactivation using thiomersal at final concentration of 0.04%, n = 3

After inactivation cell were sedimented by centrifugation at 3,000 g within 20 min at 4 °C, and the sediment was re-suspended in pH 7.2 phosphate buffer solution up to 100 unit concentration (10^{10} m.c./cm^3) using turbidity

Table
Haemagglutinating activity of *A. paragallinarum* antigens in HA after inactivation with different agents

Inactivant	Concentration, %	Antigen titre (log_2) after storage, months					
		0	2	4	6	8	10
Formalin	0.10	7.2 ± 0.2	7.3 ± 0.2	7.2 ± 0.2	7.0 ± 0.2	6.8 ± 0.1	6.8 ± 0.1
Thiomersal	0.04	8.4 ± 0.1	8.2 ± 0.1	7.4 ± 0.1	6.4 ± 0.1	5.8 ± 0.2	5.2 ± 0.2

meter. The antigens obtained were stored at +4 °C during 10 months (period of study).

The final stage of the studies consisted in the determination of haemagglutinating activity of *A. paragallinarum* antigens after inactivation with different agents (Table).

Haemagglutinating activity of the antigen, inactivated with thiomersal, was higher immediately after preparation than of the formalin-inactivated one ($P < 0.05$). However in 10 months thiomersal-inactivated antigen activity decreased by 3.1 log_2 ($P < 0.05$), whereas formalin-inactivated antigen activity showed decrease by 0.4 log_2 ($P > 0.05$).

CONCLUSION

The studies performed demonstrate that formalin at final concentration of 0.10% at 37 °C ensures complete inactivation of *A. paragallinarum* broth culture within 4.3 ± 0.1 h. 0.04% thiomersal at 37 °C also ensured complete inactivation of bacteria within 5.8 ± 0.1 h.

To inactivate *Avibacterium paragallinarum* virulent strains it is reasonable to use formalin, because antigen exposed to formalin retains high haemagglutinating activity during 10 months (period of study) thus enabling to prepare high-quality raw materials for inactivated vaccine production.

Conflict of interest. The authors declare no conflict of interest.

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