ISSN 0003-6838, Applied Biochemistry and Microbiology, 2019, Vol. 55, No. 6, pp. 704–710. © Pleiades Publishing, Inc., 2019.
Russian Text © The Author(s), 2019, published in Prikladnaya Biokhimiya i Mikrobiologiya, 2019, Vol. 55, No. 6, pp. 617–624.

Comparative Analysis of the Efficiency of Chicken and Rabbit Antibodies in Competitive Enzyme Linked Immunoassay for the Detection of Bovine Beta-Casomorphin 7

A. A. Pechelyulko[a], Y. N. Tarakanova[a], D. A. Dmitriev[a], Y. S. Massino[a], V. Y. Kost[b], E. A. Rogozhin[b, c], O. L. Segal[a], and A. D Dmitriev[a, *]

[a] *Mechnikov Scientific Research Institute of Vaccines and Sera, Moscow, 105064 Russia*
[b] *Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Moscow, 117997 Russia*
[c] *Gause Institute of New Antibiotics, Moscow, 119021 Russia*
**e-mail: add0547@yandex.ru*

Received August 8, 2018; revised March 19, 2019; accepted June 20, 2019

Abstract—Rabbit and chicken (IgY) polyclonal antibodies were compared with respect to their performance in competitive enzyme linked immunoassays (ELISA) for the determination of opioid peptide β-casomorphin 7 (BCM-7) released from variant A1 of bovine β-casein. To obtain antibodies, the animals (four rabbits and four hens) were immunized (with similar regimes) with BCM-7 conjugated to bovine serum albumin. Comparison of the binding curves of biotinylated BCM-7 obtained with affinity-purified mammalian and avian antibodies immobilized on the surface of polystyrene microtiter immunoplates via passive adsorption (in optimal conditions) showed that rabbit antibodies captured biotinylated antigen 100 times more efficiently than hen antibodies within the given antibody panel. The most efficient rabbit antibodies were used to construct a highly sensitive, competitive ELISA for the detection of BCM-7 (the minimal detection limit was 0.2 ng/mL). The chicken antibodies proved unsuitable for such use due to their low affinity. These results indicate that it is necessary to make comparisons with methods based on mammalian antibodies in the construction of quantitative ELISA based on chicken polyclonal antibodies.

Keywords: chicken antibodies, bovine β-casomorphin 7, IgY technology
DOI: 10.1134/S0003683819060103

INTRODUCTION

In recent years, researchers have repeatedly proposed the widespread use of chicken IgY polyclonal antibodies (PCAs) as components in immunochemical analytical (diagnostic) assays instead of PCAs and, in some cases, monoclonal antibodies (MCAs), which are generated via mammal immunization [1–3]. In this case, the following potential advantages of such substitution were usually mentioned. IgY does not bind to rheumatoid factor and Fc receptors; IgY does not activate the mammalian complement system, and nonspecific binding in immunodiagnostic systems is reduced. Other important advantages are the relative cheapness of the method and the bioethical benefits related to the high yield of IgY from hen egg yolks [1–3].

In addition, it was speculated that chicken PCAs may facilitate the creation of immunochemical assays to determine antigens that elicit a weak immunoresponse in mammals or those that do not provoke a response. It is believed that the large phylogenetic distance between hens and mammals can cause high immunoreactivity during the immunization of hens with mammalian antigens (due to the small homology of mammalian antigens with hen proteins); hence, it is possible to generate antibodies with wider epitope specificity. Furthermore, chicken antibodies have reduced cross-reactivity with mammalian proteins [1–3].

New opportunities arrive in the production of recombinant chicken MCAs [4, 5]. Technically, it is a simpler task than the production of recombinant mammalian MCAs, since the primary structure of the IgY molecule possesses certain features. Thus, PCR amplification of the chicken antibody light and heavy chain sequences requires a significantly lower number of primers than in the case of mammalian antibodies [4]. It is also believed that IgY antibodies are more efficient for use in the humanization process (the replacement of chicken sequences with human sequences), which is a necessary process when antibodies are used in immunotherapy [4]. Therefore, all of this led to the appearance of biotechnological companies that specialize in the production of monoclonal recombinant IgY antibodies for the creation of new efficient immunodiagnostic systems and immunotherapeutic products.

It shoud be noted that the impressive success of recombinant MCA technologies does not obviate the need to obtain (when possible) traditional natural antibodies (with the preserved native structure of IgY) for immunochemical assays. The production of chicken MCAs with native structure is possible in theory with the classic hybridoma technology (by the fusion of an antibody-producing B cell from immunized chickens with a hen myeloma cell). However, chicken hybridomas were unstable (in contrast to mouse and rat hybridomas), which made the widespread use of this approach significantly difficult [4]. Therefore, the most technically affordable and economical way to generate native chicken antibodies for immunochemical assay and immunodiagnosis at present is hen immunization with subsequent extraction of PCAs from egg yolks. Over the years, articles have reported the use of chicken antibodies to assay a range of antigens: tumor markers [6, 7], hormones [8], viral proteins [9–11], pathogenic bacteria [12–14], protozoa [15], helminths [16], prions [17], and other biomolecules [2]. This may give the impression of simplicity in the replacement of mammalian antibodies with chicken PCAs in many assays. At the same time, it is known that IgY and IgG molecules differ significantly in structure, because IgG molecules lack the hinge region that would provide flexibility [1–3]. The phylogenetic distance between hens and mammals, which causes differences in the immune system, can be not only an advantage of technology; it can also create obstacles to the recognition of some antigens by the immune system [5]. Therefore, a more nuanced study of the efficiency of chicken PCAs compared to mammalian antibodies is essential when IgY is used in immunochemical assays. We previously studied the possibility of the replacement of mammalian PCAs and MCAs with chicken antibodies in solid-phase enzyme-linked immunosorbent assay (ELISA) of the hepatitis B virus surface antigen (HBsAg) [18]. We succeeded in the generation of efficient chicken PCAs to HBsAg and were able to use them instead of immobilized mouse MCAs in the sandwich assay of HBsAg and to maintain the high analytical sensitivity of ELISA (although it was slightly reduced compared to the original version).

In addition, it was important to assess chicken antibodies in comparison with mammalian antibodies when used in another variant of ELISA, i.e., in the competitive assay.

The goal of this work was to compare the efficiency of chicken antibodies and rabbit PCAs in competitive ELISA in the determination of β-casomorphin-7 (BCM-7), an opioid peptide (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) formed from cow β-casein type A1 [19].

MATERIALS AND METHODS

Materials. The materials were the MaxiSorp 96-well polystyrene ELISA immunoplates of modular strips design (Nunc, Denmark), BCM-7 (Sigma, United States), streptavidin-peroxidase (Biosource, United States), EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Fisher Scientific, United States), BrCN-sepharose (Farmacia, Sweden), a solution of tetramethylbenzidine (TMB) (NPO Diagnostic systems, Russia) and other reagents (Sigma, United States).

Preparation of biotinylated casomorphin. Biotinylated casomorphin was obtained with two methods.

Method 1. Eight milliliters of LC-LC-biotin (2 mg/mL) dissolved in buffer 0.1 M $NaHCO_3$, pH 8.0, was added to 1.6 mL of BCM-7 solution (2 mg/mL) in 0.1 M $NaHCO_3$, pH 8.0. The reaction was run for 2 h on an ice bath. Biotinylated peptide was purified via high-performance liquid hydrophobic-interaction chromatography (Agilent Technologies, United States) according to the manufacturer's recommendations.

Method 2. Biotinylated casomorphin was produced by solid-phase synthesis in Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry (Moscow).

High-performance liquid chromatography analysis. The products of the biotinylation reaction were purified via reverse-phase high performance liquid chromatography (HPLC). The reaction products diluted in 1 mL of 25 mM Na-phosphate buffer were injected into a XBridge C18 BEH column (5 μm particle size, 130 Å, 4.6 × 250 mm, Waters, Ireland) equilibrated with 5% solvent B (80% CH_3CN/0.1% trifluoroacetic acid). After the elution of unbound compounds from the column, the bound components were separated in a linear gradient of an increasing concentration of solvent B from 5 to 50% over 60 min at a flow rate of the mobile phase 0.9 mL/min. The components were detected at 214 nm.

Antibody production, affinity purification and antibody activity estimation. We used the methods described previously [18, 19]: (1) the immunization of animals with BCM-7 conjugated to bovine serum albumin (BSA), (2) the determination of antibodies in the immune sera of rabbits and hen egg yolks, (3) the preparation of affine carriers, (4) the affine purification of antibodies from rabbit sera and hen egg yolks, (5) and the determination of the antigen-binding activity of affine-purified antibodies with adsorbed antigen, and (6) the antigen-binding activity of adsorbed antigens with a soluble antigen labeled by biotin. The parameter that characterizes the binding of BCM-7-biotin with immobilized antibodies was the analytical sensitivity of the method, estimated as the tangent of the angle of slope of the linear part of the binding curve to the concentration axis (the slope coefficient of the curve indicated in this work by the letter symbol *k*) [20–22].

Optimization of conditions for adsorption of the tested antibodies. In this paper, the conditions for the adsorption of affine-purified antibodies, which provided their maximum binding to the antigen in the solution, were considered the optimal conditions.

Optimization of adsorption pH. Antibodies (100 μL per a well) were adsorbed onto the surface of immuno plates for 16–20 h at room temperature with an excess amount of antibodies (10 μg/mL) at the first step. Adsorption was performed with the following buffers: 0.1 M glycin-HCl-buffer, pH 2.8; 0.025 M Na-phosphate buffer, pH 7.5; and 0.05 M Na-bicarbonate buffer, pH 9.5. The antigen-binding activity of the adsorbed antibodies was assessed by incubation with serial dilutions of biotinylated BCM-7 followed by incubation with streptavidin-peroxidase and staining with TMB, to construct binding curves, as described previously [22].

Determination of the optimal quantity of antibodies adsorbed on the surface of immunoplates. The optimal quantity of adsorbed antibodies on immunoplates was assessed as described previously at optimal pH for adsorption (see the previous section). The optimal quantity of adsorbed antibodies was 6 μg/mL for rabbit antibodies and 12 μg/mL for hen antibodies (with insignificant deviations from the indicated values) [18].

Assay of BCM-7 by competitive ELISA. BCM-7 was assayed by competitive ELISA according to a previously described procedure with modifications [19]. Antibodies were adsorbed onto the surface of immunoplates (6 μg/mL of rabbit antibodies and 12 μg/mL of hen antibodies in optimal buffer) for 16–20 h at room temperature. After washing, 50 μL of a BCM-7-biotin solution (2 ng/mL for rabbit and 300 ng/mL for hen antibodies in ELISA buffer) were loaded into each well, and 50 μL of BCM-7 solutions were then added with an increasing quantity from 0 to 1000 μg/mL. The mixture was incubated over 2 h at 37°C. After washing, the immunoplates were incubated with streptavidin-peroxidase and stained with TMB as described previously [18, 22].

RESULTS AND DISCUSSION

The immune response after the immunization of animals with BCM-7 conjugated to BSA. We performed a comparative analysis of the efficiency of affine-purified rabbit and chicken antibodies in competitive solid-phase ELISA. The selected antigen was BCM-7, the peptide which is the fragment of β-casein from cow's milk type A1 and has the properties of opioid peptides [19]. We previously successfully created a highly sensitive enzyme immunoassay to determine this ligand with rabbit PCAs [19], and we relied on our experience in this paper. BCM-7 conjugated to BSA was used as the immunogen. The immune response was assessed from the binding of rabbit serum antibodies or hen egg yolk antibodies to immobilized BCM-7. After five to six rounds of immunization, all animals (four hens and four rabbits) produced antibodies to BCM-7. The antibodies were affine-purified with a column with CNBr-activated sepharose conjugated to BCM-7. The coefficients for the binding curve of affine-purified rabbit antibodies to immobilized BCM-7 varied from 0.053 to 0.125, i.e., they differed by 2.4 times (the curves of the antibody binding to immobilized antigen are not given). Antibodies of rabbit no. 1 ($k = 0.1250$) were the most efficient in binding the immobilized antigen. The curve coefficients expressing the binding of chicken antibodies were significantly lower and within the limits of 0.0012–0.004. However, based on this preliminary assessment, it was not possible to compare the antigen-binding activity of chicken and rabbit antibodies, since antihen and anti-rabbit conjugates, which were used for the detection of immune complexes, can vary significantly according to their activity. Nevertheless, it can be concluded that both rabbit and chicken antibodies elicited an immunoresponse after immunization with BCM-7. Affinity-purified antibodies of rabbit no. 1 and affinity-purified antibodies of hen no. 4 showed the greatest binding activity to the immobilized antigen.

Determination of the optimal conditions for adsorption of affine-purified rabbit and chicken antibodies on the surface of immunoplates. Panels with affine-purified chicken and rabbit PCAs, which were the most efficient in binding to the labeled antigen after adsorption to the solid phase under optimal conditions, were proposed for the comparison of competitive enzyme immunoassays to determine BCM-7 with chicken and mammalian antibodies. The conditions for antibody adsorption (pH of adsorption and saturating concentration) that provided their maximum antigen-binding activity were regarded as optimal. The parameter characterizing the efficiency of antibody binding to the antigen was the analytical sensitivity of the method estimated as the slope ratio of the linear part of the binding curve to the concentration line (the slope coefficient of the curve was indicated with the letter symbol k) [20–22]. This is one of the methods used to evaluate and compare the efficiency of ELISA during optimization of the procedure [20, 23].

The preservation of the high antigen-binding activity of adsorbed antibodies, along with affinity, represents a factor that provides the maximal sensitivity of the analysis in competitive solid-phase ELISA [24].

Therefore, the initial stage of the comparison of the properties of chicken and rabbit PCAs involved optimization of the conditions for adsorption onto the surface of polystyrene immunoplates for each of the eight generated PCAs. In actual practice, two factors are commonly varied: the saturating antibody concentration and the pH of the buffer used for saturation. The optimal saturating concentrations of affine-purified antibodies were 6 μg/mL for rabbit antibodies and 12 μg/mL for egg yolk antibodies (with insignificant deviations).

At the next stage, the optimal pH for the adsorption of affine-purified antibodies was identified. Phosphate and carbonate buffers at pH 7.5 and 9.5 have traditionally been regarded as the optimal buffer systems for antibody adsorption onto the immunoplate surface. At the same time, previous papers described the

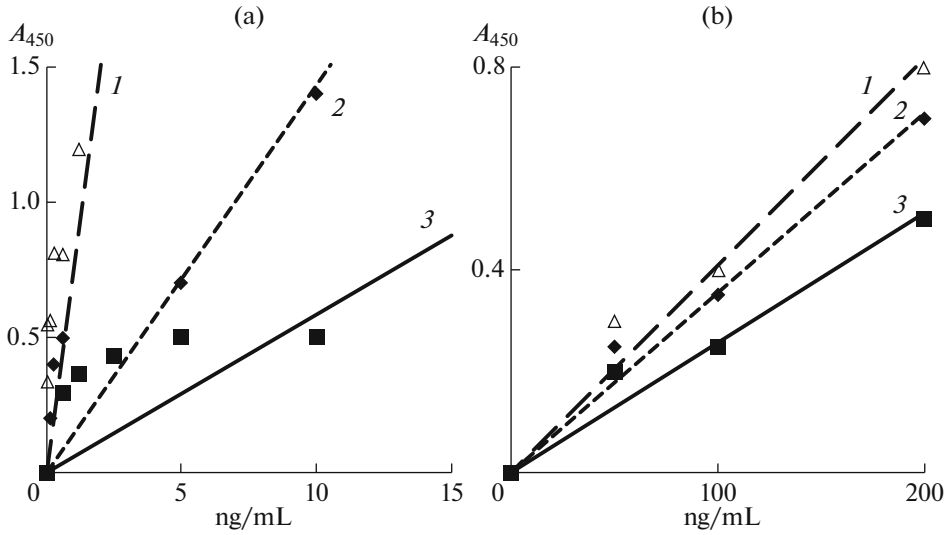

Fig. 1 Binding of biotinylated β-casomorphin-7 (ng/mL) with affine-purified antibodies of (a) rabbits and (b) hens: a—antibody adsorption at pH 7.5 (*1*) $y = 0.76x$, at pH 9.5 (*2*) $y = 0.14x$, at pH 2.8 (3) $y = 0.06x$; b—antibody adsorption at pH 7.5 (*1*) $y = 0.0042$, at pH 9.5 (*2*) $y = 0.0037$, at pH 2.8 (*3*) $y = 0.0026$.

mammalian and chicken antibodies that possessed the maximal antigen-binding activity after adsorption under harsher pH conditions of 2.8 [18, 21, 22]. The pH values that favored the maximal antigen-binding activity of the tested antibodies after adsorption were determined. Buffers with the following pH values were used for adsorption: 2.8, 7.5, and 9.5 (for the composition of the buffers, see Materials and Methods). The binding curves of biotinylated BCM-7 with adsorbed antibodies were constructed after immunoplate saturation. Table 1 summarizes the data on the optimal adsorption pH values. It was found that the optimal adsorption pH of rabbit antibodies no. 1, 2, and 3 was 7.5; for one of the tested antibodies (No. 4), two optimal adsorption pH values (pH 7.5 and 9.5) were observed. At the same time, the antibodies of three hens (No. 1, 2, and 3) were equally efficient in binding biotinylated casomorphin after adsorption at a pH of 7.5 and pH 9.5 (Table 1). The antibodies of one hen (No. 4) demonstrated the maximal binding of biotinylated BCM-7 after immobilization at two pH values: 7.5 and 2.8 (Table 1).

The described experiments are illustrated in Fig. 1, which presents the binding curves of biotinylated BCM-7 with affine-purified chicken antibodies (Fig. 1b) adsorbed at mentioned pH values. Similar curves for rabbit antibodies are shown in Fig. 1a, which illustrates the degree to which the antigen-binding activity depended on the pH of antibody adsorption. Thus, after adsorption at a pH of 7.5, the coefficient of the binding curve was 0.76. Upon the binding of BCM-7-biotin after the adsorption of rabbit antibodies at pH 2.8, the coefficient of the curve decreased by 13-folds ($k = 0.06$).

Binding efficiency of biotinylated BCM-7 with affine-purified rabbit and chicken antibodies. Table 2 summarizes the coefficients of the binding curves of BCM-7-biotin with antibodies. The data show that the binding of biotinylated BCM-7 to chicken antibodies was much less efficient than that with rabbit antibodies. Thus, the binding of the best chicken antibodies, no. 2 ($k = 0.040 \pm 0.002$, Table 2), to BCM-7-biotin was 190 times less efficient than that of the best rabbit antibodies, no. 1 ($k = 7.62 \pm 0.13$). The binding of the best chicken antibodies, no. 2, was also 13 times less efficient than the binding activity observed for the worst rabbit antibodies, no. 3.

In general, the binding efficiency of adsorbed chicken antibodies to BCM-7-biotin was 100 times lower than that of rabbit antibodies (based on the average values of the binding curve coefficients). The average value of the curve coefficients for binding to biotinylated BCM-7 was ≈0.024 for chicken antibod-

Table 1. pH values for the adsorption of affine-purified hen and rabbit antibodies leading to maximal binding with biotinylated β-casomorphin-7

Animal number	Optimal pH for adsorption of the tested antibodies	
	rabbits	hens
1	7.5	7.5/9.5*
2	7.5	7.5/9.5*
3	7.5	7.5/9.5*
4	7.5/9.5 *	7.5/2.8*

* The coefficients of the binding curves for the two indicated values of pH coincided.

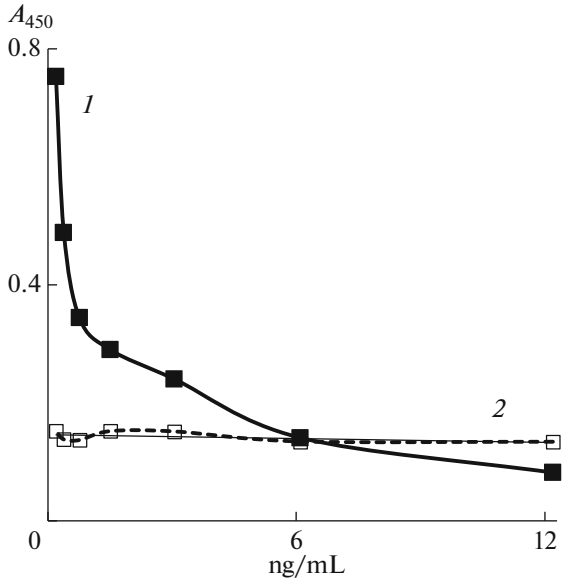

Fig. 2. Competitive method for the determination of β-casomorphin-7 with affine-purified antibodies of rabbits and hens adsorbed onto the surface of immunoplates under optimal conditions. *1*—rabbit PCAs, *2*—hen PCAs. The equation of the logarithmic curve $y = -0.145\ln(x) + 0.3944$; $R^2 = 0.9072$.

ies and ≈2.50 for rabbit antibodies. It should be taken into account that twice as many chicken antibodies (12 μg/mL) as rabbit antibodies (6 μg/mL) were adsorbed onto immunoplates in these experiments.

Comparison of the efficacy of chicken and rabbit antibodies in competitive ELISA determination of BCM-7. To construct competitive calibration curves for the determination of β-casomorphin-7, we chose the antibodies that had the maximum antigen binding activity after adsorption under optimal conditions: the antibodies of hen no. 2, adsorption optimum—pH 7.5 ($k = 0.04 \pm 0.002$), and the antibodies of rabbit no. 1, adsorption optimum pH 7.5 ($k = 7.62 \pm 0.13$). The results are shown in Fig. 2. According to Fig. 2, the use of rabbit PCAs made it possible to obtain a qualitative calibration curve within a BCM-7 concentration range of 0.2–12 ng/mL between 90 and 10% inhibition of biotinylated β-casomorphin-7 binding to immobilized antibodies. The minimum determined concentration (the reduction of absorption at the tested concentration relative to the absorption of zero standard ±3 standard deviations) of BCM-7 was 0.2 ± 0.016 ng/mL, which approximately corresponded to the result of a previous work [19]. However, the curve obtained with chicken antibodies could not be considered suitable for the determination of the concentration of the tested antigen due to the low performance of chicken PCAs immobilized on a solid phase in binding BCM-7 (even though these antibodies were also adsorbed under optimal conditions).

Table 2. Coefficients of the binding curves for biotinylated casomorphin-7 with antibodies adsorbed onto the surface of immunoplates under optimal conditions

Animal number	Coefficients of the binding curves for adsorbed antibodies with biotinylated β-casomorphin-7 ($k \times 10 \pm$ confidence interval, $n = 6$)	
	rabbit antibodies	hen antibodies
1	7.62 ± 0.13	0.020 ± 0.001
2	0.61 ± 0.03	0.040 ± 0.002
3	0.53 ± 0.03	0.022 ± 0.001
4	1.25 ± 0.01	0.012 ± 0.001
Average	2.50	0.024

The study of the opioid peptide BCM-7 attracted interest in the 1980s, when it was suggested based on a range of studies (some of which were performed by large dairy companies in New Zealand) that this peptide may increase the risk of diabetes, cardiovascular disease, childhood autism, and other diseases [25]. BCM-7 is released in the gastrointestinal tract after the cleavage of milk A1 β-casein. The splitting of milk A2 β-casein does not result in the formation of BCM-7. A1 and A2 proteins are found in different proportions in cow's milk depending on the breed of cattle. In Asia and Africa, cows lack a form of A1 β-casein, and the variety of cow milk containing only type A2 β-casein is referred to as milk A2. However, in other regions, such as Europe, United States, and New Zealand, cow's milk contains both types of casein: A1 and A2 [25]. Due to the hypothesized risk to human health posed by the BCM-7 peptide, a range of dairy companies began to actively advertise milk A2 and milk A2–based products. However, the concerns about BCM-7 were not sufficiently confirmed by subsequent studies. Nonetheless, it was suggested that BCM-7 may have an adverse effect on the digestive system, leading to milk intolerance. A group of experts from Germany and Hungary working within the framework of the Cochrane Collaboration recently conducted a new analysis of the literature data related to the health effects of the BCM-7 peptide. According to their review published in 2019, there is a moderate probability that BCM-7 formed by the consumption of A1 milk can cause adverse effects on digestion, but the relationship of this peptide with other diseases is very unlikely. Nevertheless, the authors of the review concluded that this issue requires further investigation [25]. Hence, there is a need for efficient ELISA methods to assay BCM-7, which will be based on both mammalian and chicken antibodies (if the latter is possible).

Our search of the literature data yielded only one work (published in 1998 by researchers from Canada) in which the authors also obtained chicken PCAs specific to BCM-7 and described their properties [26]. A more nuanced content of the study is provided in the thesis [27] prepared by one of the coauthors (the

results of which were used in the preparation of the mentioned paper) [26]. The study describes a competitive method for the determination of BCM-7 based on chicken antibodies. The authors did not attempt to compare the efficiency of hen and mammalian PCAs; the task was to develop a competitive method for the determination of this antigen. At the same time, Canadian researchers used a slightly different format (as compared to our work) of the competitive method: the antigen (BCM-7) was adsorbed onto solid phase instead of PCA against BCM-7. This format (which is commonly perceived as possessing a slightly lower sensitivity) allowed the authors to eliminate minor amounts of PCAs to BSA via washing [27] (we did not encounter such a problem in the method that we used). The authors succeeded in developing a competitive method for the determination of BCM-7 based on chicken PCAs with a minimal detection dose of 5 ng/mL; nevertheless, as reported in this work, the immunoresponse was elicited only in one of the six immunized hens.

Although all hens in our work developed an immunoresponse, the response was much weaker than in rabbits. In addition, purified chicken PCAs were not sufficiently efficient for the creation of a competitive enzyme immunoassay to determine BCM. At the same time, we easily reproduced the results of our previous work on rabbits, which described the competitive ELISA for the determination of BCM-7 [19] and developed another competitive method (based on rabbit PCAs) with binding curves that make it possible to measure the antigen in approximately the same concentration range. Such PCAs may be of interest for research on the physiological effects of BCM-7 in experiments (e.g., in laboratory animals) and in humans (it was shown that BCM-7 can not only be released in the gastrointestinal tract after milk consumption but it can be absorbed into the circulatory system and other organs and tissues, e.g., in children) [19, 25]. In addition, some authors made attempts to use BCM-7 antibodies for the detection (in combination with peptide purification methods) of minor amounts of this peptide in dairy products [28].

It was proposed that the phylogenetic distance between hens and mammals favors the elicitation of a strong immunoresponse to a range of mammalian protein antigens due to the absence of similar structures and molecules in hens. However, according to our work and, partly, the work of Canadian authors (in which the immunoresponse was observed in only one of six hens), this assumption cannot be considered universal. It is known that hens have a less efficient antigen presentation system, which can lead to a complete lack of immunoresponse to a number of agents depending on the haplotype of the main histocompatibility complex (MHC) [5, 28]. With regard to an antigen such as BCM-7, with consideration of the phenomenon of immunological tolerance, including to β-casein [29], it is noted that BCM-7 by its primary structure is also a quite foreign antigen for rabbits. Even though mammalian β-casein genes have a significant homology, the site where the BCM-7 peptide is cleaved shows a significant divergence. For example, while the β-casein molecule of cow and human milk contains the characteristic amino acid sequence Tyr-Pro-Phe [30] in corresponding area, rabbits retain only two amino acids, Pro-Phe, of the common sequence [31].

Meanwhile, in a recent work, we generated IgY antibodies to hen HBsAg, which showed a high efficiency in sandwich assay for the determination of HBsAg; nonetheless, it was slightly lower (30%) than that of reference MCAs to this viral protein [18].

CONCLUSIONS

Thus, it seems that the replacement of mammalian antibodies with IgY antibodies is unlikely to be a generally applicable approach, as it seemed at the beginning of the implementation of IgY technology. However, it provides another useful alternative in the creation of highly efficient antibodies for the development of a range of immunochemical assays, especially in combination with modern methods of recombinant antibody generation that make it possible to increase their affinity artificially [4, 5].

This paper compares the antigen-binding properties of antibodies specific to BCM-7 obtained from four hens and four rabbits immunized with this peptide. The immunoresponse of hens to the antigen was much weaker than that of rabbits, and the efficacy of chicken PCAs was too low to create a competitive method for the determination of BCM-7. At the same time, it was possible to create a test system (solid-phase competitive ELISA) with rabbit PCAs for the determination of BCM-7 with a high analytical sensitivity, which can be useful for the analysis of BCM-7. In light of the literature data on the generation of hen PCAs to various antigens and the experience of the authors [18] in generating highly efficient chicken PCAs to HBsAg, the results of the work do not cast doubt on the usefulness of chicken PCAs for the design of solid-phase ELISA. However, they indicate the need to compare the efficiency of test systems based on hen PCAs in the case of each specific antigen with methods based on the use of mammalian antibodies (rabbits, mice MCAs, etc.).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

Statement on Animal Welfare. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

REFERENCES

1. Schade, R., Calzado, E.G., Sarmiento, R., Chacana, P.A., Porankiewicz-Asplund, J., and Terzolo, H.R., *Altern. Lab. Anim.*, 2005, vol. 33, no. 2, pp. 129–154.
2. Tambourgi, D.V., *Vet. Immunol. Immunopathol.*, 2010, vol. 135, nos. 3–4, pp. 173–180.
3. Spillner, E., Braren, I., Greunke, K., Seismann, H., Blank, S., and Plessis, D., *Biologicals*, 2012, vol. 40, no. 5, pp. 313–322.
4. Lee, W., Syed, AtifA., Tan, S.C., and Leow, C.H., *J. Immunol. Methods*, 2017, vol. 447, pp. 71–85.
5. Júnior, F.Á., dos Pacheco, S.J., de Oliveira, S.I., Alves, M.A., Leonel, E.G., and Rodrigues, R.I., *Ciencia Rural*, 2018, vol. 48, no. 8. https://doi.org/10.1590/0103-8478cr20180250
6. Łupicka-Słowik, A., Walczak, M., Grzywa, R., Bobrek, K., Łęcka, M., Boivin, S., Gaweł, A., Stefaniak, T., Oleksyszyn, J., and Sieńczyk, M., *Bioanalysis*, vol. 6, no. 23, pp. 3197–3213.
7. Xiao, Y., Gao, X., Gannot, G., Emmert-Buck, M.R., Srivastava, S., Wagner, P.D., Amos, M.D., and Barker, P.E., *Int. J. Cancer*, 2008, vol. 122, no. 10, pp. 2178–2186.
8. Kuronen, I., Kokko, H., Mononen, I., and Parviainen, M., *Eur. J. Clin. Chem. Clin. Biochem.*, 1997, vol. 35, no. 6, pp. 435– 440.
9. Di Lonardo, A., Marcante, M.L., Poggiali, F., Hamsoikova, E., and Venuti, A., *Arch. Virol.*, 2001, vol. 146, no. 1, pp. 117–125.
10. de Paula, V.S., Silva AdosS., de Vasconcelos, G.A., Iff, E.T., Silva, M.E., Kappel, L.A., Cruz, P.B., and Pinto, M.A., *J. Virol. Methods*, 2011, vol. 171, no. 1, pp. 102–106.
11. Nafea, N.M., Sabbah, M.A., Al-Suhail, R., Mahdavi, A.H., and Asgary, S., *Adv. Biomed. Res.*, 2015, vol. 4, p. 100. https://doi.org/10.4103/2277-9175.156678
12. Almeida, C.M.C., Quintana-Flores, V.M., Medina-Acosta, E., Schriefer, A., Barral-Netto, M., and Dias da Silva, W., *Scand. J. Immunol.*, 2003, vol. 57, no. 6, pp. 573–582.
13. Chalghoumi, R., Théwis, A., Portetelle, D., and Beckers, Y., *Poult. Sci.*, 2008, vol. 87, no. 1, pp. 32–40.
14. Meenatchisundaram, S., Shanmugam, V., and Anjali, V.M., *J. Basic. Clin. Pharm.*, 2011, vol. 2, no. 2, pp. 109–114.
15. Ferreira Júnior, Á., Santiago, F.M, Silva, M.V, Ferreira, F.B, Macêdo Júnior, A.G, Mota, C.M, Faria, M.S, Silva Filho, H.H., Silva, D.A, Cunha-Júnior, J.P., Mineo, J.R., and Mineo, T.W., *PLoS One*, 2012, vol. 7, no. 7. e40391. https://doi.org/10.1371/journal.pone.0040391
16. Lei, J.H., Su, B.T., Xu, H., Shen, J.L., Guan, X.H., Feng, Z.Q., Li, Y.L., Xu, M.X., and Liu, W.Q., *Am. J. Trop. Med. Hyg.*, 2011, vol. 85, no. 6, pp. 1054–1059.
17. Matsuda, H., Mitsuda, H., Nakamura, N., Furusawa, S., Mohri, S., and Kitamoto, T., *FEMS Immunol. Med. Microbiol.*, 1999, vol. 23, no. 3, pp. 189–194.
18. Pechelyulko, A.A., Tarakanova, Yu.N., Dmitriev, D.A., Massino, Yu.S., Segal, O.L., Lavrov, V.F., and Dmitriev, A.D., *Appl. Biochem. Microbiol.*, 2017, vol. 53, no. 1, pp. 114–122.
19. Kost, N.V., Sokolov, O.Y., Kurasova, O.B., Dmitriev, A.D., Tarakanova, J.N., Gabaeva, M.V., Zolotarev, Y.A., Dadayan, A.K., Grachev, S.A., Korneeva, E.V., Mikheeva, I.G., and Zozulya, A.A., *Peptides*, 2009, vol. 30, no. 10, pp. 1854–1860.
20. Pardue, H.L., *Clin. Chem.*, 1997, vol. 43, no. 10, pp. 1831–1837.
21. Dmitriev, A.D., Tarakanova, J.N., Yakovleva, D.A., Dmitriev, D.A., Phartooshnaya, O.V., Kolyaskina, G.I., Massino, Y.S., Borisova, O.V., Segal, O.L., Smirnova, M.B., Ulanova, T.I., and Lavrov, V.F., *J. Immunoassay Immunochem.*, 2013, vol. 34, no. 4, pp. 414–437.
22. Tarakanova, Yu.N., Dmitriev, A.D., Massino, Yu.S., Pechelyul'ko, A.A., Segal, O.L., Skoblov, Yu.S., Ulanova, T.I., Lavrov, V.F., and Dmitriev, D.A., *Appl. Biochem. Microbiol.*, 2015, vol. 51, no. 4, pp. 462–469.
23. Li, D., Cui, Y., Morisseau, C., Gee, S.J., Bever, C.S., Liu, X., Wu, J., Hammock, B.D., and Ying, Y., *Anal. Chem.*, 2017, vol. 89, no. 11, pp. 6248–6256.
24. Butler, J.E., in *Methods in Molecular Medicine: Molecular Diagnosis of Infectious Diseases*, Decker, J. and Reischl, U., Eds., Totowa, N. J.: Humana Press Inc., 2004, pp. 333–372.
25. Küllenberg de Gaudry, D., Lohner, S., Schmucker, C., Kapp, P., Motschall, E., Hörrlein, S., Röger, C., and Meerpohl, J.J., *Nutr. Rev.*, 2019, vol. 77, no. 5, pp. 278–306.
26. Yannakis, J. and Ozimek, L., *Milchwissenschaft*, 1998, vol. 53, no. 8, pp. 436–440.
27. Yannakis, J., Development of IgY antibodies in egg-yolk against beta-casomorphin-7, *A Thesis Submitted to the Faculty of Graduate Studies and Research for the Degree of Master of Science*, Canada, University of Alberta, 1997.
28. Kaufman, J., *Trends Immunol.*, 2018, vol. 39, no. 5, pp. 367–379.
29. Derbinski, J., Pinto, S., Rosch, S., Hexel, K., and Kyewski, B., *Proc. Natl. Acad. Sci. U. S. A.*, 2008, vol. 105, no. 2, pp. 657–662.
30. Enjapoori, A.K., Kukuljan, S., Dwyer, K.M., and Sharp, J.A., *Nutrition*, 2019, vol. 57, pp. 259–267.
31. Lonnerdal, B., Bergstrom, S., Andersson, Y., Hjalmarsson, K., Sundqvist, A.K., and Hernell, O., *FEBS Lett.*, 1990, vol. 269, no. 1, pp. 153– 156.

Translated by M. Novikova