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Determination of indicators for tests of polysept (polyhexamethylene guanidine hydrochloride) for flocculation properties

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SUMMARY

In vaccine production, it is particularly important to purify the virus-containing suspension in order to remove ballast proteins and fats, which, when present in high concentrations, are responsible for depression or allergic reactions in animals. Polyguanidine and its derivatives have long been used for such purposes. At present, the market offers polyhexamethylene guanidine hydrochloride, a cationic polyelectrolyte with a unique combination of physico-chemical and biocidal properties allowing for it to be used in nearly all spheres of economy. Flocculation properties of polysept (polyhexamethylene guanidine hydrochloride) vary from batch to batch, and this has necessitated the development of a test system for determination of the incoming material quality, which has a significant impact on virus antigen concentration during vaccine production. Seven batches of polyhexamethylene guanidine were tested for flocculation properties, changes in FMDV immunogenic component concentration in the virus-containing suspension, osmolality of solutions at different percentage concentrations. Indicators of incoming material suitability for FMD vaccine production were determined. The batches of polysept should be tested for flocculation properties at different concentrations of the polymer (0.007, 0.0105 and 0.01575%) in dynamics during 24 hours. After this period, the turbidity of solutions should not exceed 30 FNU (formazin turbidity) at concentrations of 0.0105 and 0.01575%. It is also necessary to determine the osmolality of polysept solutions at different percentage concentrations (6, 8, 10, 12, 14%). Osmolality values should be within the following ranges: 260 ± 20 mOsm for a 6% solution; 330 ± 25 mOsm for an 8% solution; 400 ± 25 mOsm for a 10% solution; 460 ± 30 mOsm for a 12% solution; 520 ± 20 mOsm for a 14% solution.

Keywords: polyhexamethylene guanidine hydrochloride, foot-and-mouth disease virus antigen, immunogenic components, flocculation, turbidity, osmolality

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Определение критериев для исследования флокулирующих свойств полисепта (полигексаметиленгуанидин гидрохлорида)

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РЕЗЮМЕ

При производстве вакцин важную роль играет очистка вирусной суспензии от балластных белков и жиров, высокая концентрация которых вызывает угнетение организма животных или аллергические реакции. На протяжении длительного времени для этих целей применяли полигуанидин и его производные. В настоящее время на рынке предлагают катионный полиэлектролит полигексаметиленгуанидин гидрохлорид, обладающий уникальным сочетанием физико-химических и биоцидных свойств, которые позволяют использовать его практически во всех сферах народного хозяйства. Партии полисепта (полигексаметиленгуанидин гидрохлорида) отличаются друг от друга по флокулирующим свойствам, поэтому возникла необходимость разработать тест-систему для определения качества поступающей продукции, существенно влияющего на потерю антигена вируса при производстве вакцин. Были изучены как флокулирующие свойства, так и потеря иммуногенных компонентов вируса ящура из вируссодержащей суспензии, а также осмоляльность растворов разной процентной концентрации семи серий полигексаметиленгуанидин гидрохлорида. Установлены критерии пригодности поступающей продукции для производства противоящурных вакцин: проверка в динамике флокулирующих качеств партий полимера при разных его концентрациях (0,007; 0,0105; 0,01575%) на протяжении 24 ч. Через указанное время мутность раствора должна быть не более 30 FNU (формазиновая степень мутности) при концентрациях 0,0105 и 0,01575%. Также необходимо определять осмоляльность растворов полисепта разной процентной концентрации (6, 8, 10, 12, 14%). Значение осмоляльности должно укладываться в следующие границы: 6%-й раствор – 260 ± 20 mOsm; 8%-й – 330 ± 25 mOsm; 10%-й – 400 ± 25 mOsm; 12%-й – 460 ± 30 mOsm; 14%-й – 520 ± 20 mOsm.

Ключевые слова: полигексаметиленгуанидин гидрохлорид, антиген вируса ящура, иммуногенные компоненты, флокуляция, мутность, осмоляльность

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INTRODUCTION

In vaccine production, the purification of the virus-containing suspension is of particular importance. Polyguanidine and its derivatives have long been used for such purposes. At present, the market offers polyhexamethylene guanidine hydrochloride (PHMG hydrochloride), a cationic polyelectrolyte with a unique combination of physico-chemical and biocidal properties allowing for it to be used in nearly all spheres of economy [1–8].

Polyhexamethylene guanidine hydrochloride is a water-soluble chlorine-containing polymer with a molecular mass of 10,000 Da, represented by formula $[–NH–C=(NH\times HCl)–NH–(CH_2)_6–]_n$. Chlorine included in its composition is a complex salt of hydrogen chloride with a strong basic nitrogen of the compound. The preparation with the empirical formula $(C_7H_{16}N_3Cl)_n$ manufactured under the trademark "Polysept" (OOO "Pharma-Pokrov", Russia) is a water-soluble polymer product, which has the characteristics of both a cationic polyelectrolyte and a polymer, contains polar guanidine and nonpolar hexamethylene groups imparting adhesive and surfactant properties to it and can therefore be broadly applied in economy. PHMG hydrochloride has a high bactericidal and fungicidal effect. Its 0.05% solutions kill both gram-positive and gram-negative microorganisms within 5–25 minutes. The product is safe for humans, animals and the environment [8–10].

Physico-chemical properties of PHMG hydrochloride: it is colourless and odourless (some low quality product samples may smell like ammonia), fireproof, explosion-safe, fully soluble in water, alcohol-soluble, does not lose its properties at subzero temperatures, does not decompose and retains its physico-chemical and biocidal properties when heated to 120 ± 5 °C. The shelf life is at least 5 years for a 20% aqueous solution and at least 7 years for a 100% concentrate.

Biocidal properties of PHMG hydrochloride: it is a biocide with a broad-spectrum antimicrobial activity against gram-negative and gram-positive bacteria (in particular, mycobacteria causing tuberculosis and legionellosis), viruses (including enteric and post-transfusion hepatitis viruses, human immunodeficiency virus, poliomyelitis virus, influenza virus, herpesviruses, etc.), fungi, in particular mold, yeast and yeast-like fungi, fungi of the genus *Candida*, dermatophytes.

Product form: lumps (pellets) containing at least 95–98% of PHMG hydrochloride or an aqueous solution containing 20% of PHMG hydrochloride. Where necessary, aqueous solutions containing up to 50% of the active ingredient can be prepared [4].

PHMG hydrochloride is produced by the interaction of hexamethylene diamine and guanidine hydrochloride [11, 12].

Table 1
Formazin turbidity reference values

Turbidity, FNU				
< 0.10	0.1–15	16–100	101–750	> 750
ultra-low	low	medium	high	ultra-high

Polysept has been found to have flocculation properties. It is applied as a 9% solution (1.5–2.0 mg of dry matter per 1 L of waste water) [13].

In the production of inactivated vaccines against foot-and-mouth disease, polysept is added in the form of a 5 or 10% aqueous solution to reach the final concentration of 0.005–0.03% (pH 7.6–8.0). Flocculated ballast proteins are removed by centrifugation, separation or sedimentation. The use of PHMG hydrochloride concentrations greater than 0.03% results in a significant reduction of the virus concentration; a decrease in FMDV infectivity titre and 146S component concentration are observed [10, 14].

Unfortunately, flocculation properties and other characteristics of polysept may vary from batch to batch, leading to a decrease in the concentration of the virus protein used for vaccine production. It is therefore important to develop a test system for tests of PHMG hydrochloride batches for flocculation properties.

The aim of the study is the selection of a test system for tests of polysept (PHMG hydrochloride) for its flocculation properties.

MATERIALS AND METHODS

Cell line. BHK-21/SUSP/ARRIAH, a continuous suspension culture of neonatal Syrian hamster kidney cells, was used for the study [15]. The cells were grown in metal fermenters with a working capacity of up to 1,800 dm^3 in accordance with the Master formula record for production of the vaccine against FMD of various types.

PHMG hydrochloride was supplied by OOO "Pharma-Pokrov" (Russia), TU 9392-001-32963622-99, batches: No. 343 of 09 July 2020; No. 522 of 20 November 2020; No. 48 of 21 February 2021; No. 57 of 26 February 2021; No. 71 of 26 March 2021; No. 219 of 27 August 2019; No. 168 of 27 August 2021.

To perform the study, 10 and 20% solutions were prepared in an enameled container using demineralized water. The mixture was heated to 90–100 °C with constant stirring until complete dissolution of the polymer, cooled at 18–25 °C and then placed in the cold chamber (4–8 °C).

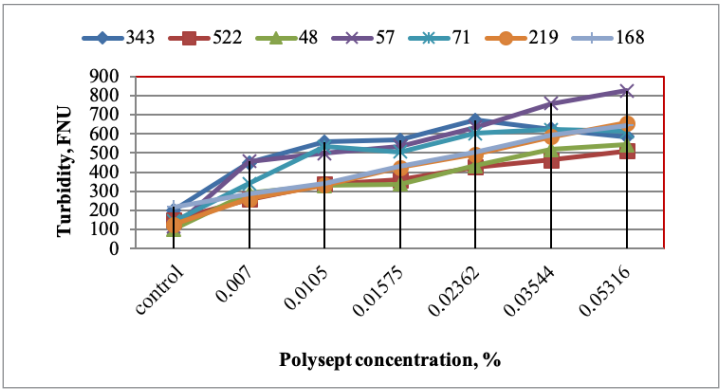

Fig. 1. Turbidity dynamics in the zero-hour sample at different polysept concentrations

The turbidity of the prepared PHMG hydrochloride solution was measured using a portable HI 98713 turbidity meter equipped with an IR-diode (Hanna Instruments, Germany) according to the manufacturer's instruction. Turbidity was reported in FNU (a formazin turbidity unit).

The values and corresponding degrees of turbidity are presented in Table 1 [16].

To measure the osmolality of the tested solutions at different concentrations (6, 8, 10, 12, 14%), an OSKR-1M cryoscopic medical osmometre (Russia) was used.

The concentration of FMDV total viral protein and its components was determined according to the "Methodical guidelines for determination of concentration of 146S, 75S, 12S components of vaccine strains of culture FMD virus with complement fixation test (CFT)" [17].

Tests for flocculation properties. The tests were carried out as follows: the inactivated FMD virus suspension was transferred into 0.5 dm^3 bottles (0.4 dm^3 of the suspension per bottle), then a 10% polysept solution was added to reach the final concentrations of 0.007, 0.0105, 0.01575, 0.02362, 0.03544, 0.05316% (1:5). Samples for turbidity tests were collected after 0 (a zero-hour sample), 2, 4, 6, 8 and 24 hours. The concentrations of total viral protein and its components were determined 24 hours after addition of polysept.

Statistical processing of data. Numerical data were statistically processed by generally accepted methods of variation statistics using a personal computer and Microsoft Excel software.

RESULTS AND DISCUSSION

During the first stages of the study, different batches of polysept were tested for their flocculation properties. For this purpose, a 10% polysept solution was added to the inactivated FMD virus suspension at different concentrations in the total volume. Samples were collected at different time intervals. It was found that the higher the flocculant percentage, the greater were turbidity values in the zero-hour samples (Fig. 1, Table 2). Turbidity in the controls varied from 101.5 ± 19.9 to 219.3 ± 10.8 FNU; in the polymer-supplemented suspension, turbidity varied from 258.0 ± 32.9 to 826.0 ± 61.6 FNU depending on the batch and concentration of polysept (the differences were significant, $p < 0.005$).

According to literature data, the adsorption of a flocculant onto dispersed phase particles can occur as a result of electrostatic, chemical interactions, ion exchange, under the effect of Van der Waals forces. Flocculation effectiveness, floc size and density depend largely on the intensity and duration of mixing, as well as on flocculant amount. The addition of a small amount of the flocculant leads to a sharp increase in floc hardness.

The formation of aggregates of particles, i.e. the binding of particles through the formation of bridges with the polymer, is a result of interaction between macromolecules adsorbed onto dispersed phase particles and loose particles. The adsorption of ionogenic flocculants onto oppositely charged dispersed phase particles occurs mainly due to electrostatic attraction. Flocculation rate is the highest when the concentration of flocculant-coated particles and that of uncoated particles are the same [18–20].

Table 2
Turbidity dynamics at different polymer concentrations ($n = 3$)

No.	PHMG batch No.	Time (hours)	Turbidity (FNU)						
			0.007%	0.0105%	0.01575%	0.02362%	0.03544%	0.05316%	Control
1	343	0	448.2 ± 12.0	557.8 ± 41.3	568.8 ± 8.6	672.8 ± 17.7	625.4 ± 34.6	585.4 ± 9.5	200.6 ± 69.5
		2	354.2 ± 9.2	238.8 ± 24.6	109.0 ± 15.0	85.6 ± 11.7	88.7 ± 3.0	69.3 ± 4.7	180.0 ± 60.0
		4	307.6 ± 12.6	117.5 ± 29.5	71.2 ± 11.4	53.3 ± 8.2	59.4 ± 5.7	49.4 ± 3.8	173.6 ± 51.3
		6	304.6 ± 11.2	85.5 ± 10.4	51.2 ± 12.8	38.2 ± 8.8	30.6 ± 8.3	19.0 ± 1.4	169.2 ± 52.7
		8	294.4 ± 14.0	70.0 ± 20.5	35.3 ± 10.6	25.8 ± 3.7	24.9 ± 2.3	19.3 ± 3.1	170.4 ± 51.8
		24	54.4 ± 22.4	47.2 ± 11.3	19.6 ± 3.5	13.1 ± 1.5	15.1 ± 2.3	12.5 ± 3.1	142.8 ± 30.1
2	522	0	258.0 ± 32.9	337.3 ± 38.8	361.0 ± 27.6	426.3 ± 29.5	464.0 ± 26.2	511.3 ± 22.3	150.7 ± 27.0
		2	223.3 ± 25.2	216.7 ± 20.8	340.7 ± 24.0	111.3 ± 3.2	85.7 ± 4.5	74.2 ± 3.3	163.0 ± 37.5
		4	184.7 ± 32.3	243.7 ± 40.5	108.7 ± 9.9	70.4 ± 5.1	58.0 ± 2.4	56.6 ± 3.6	163.7 ± 42.7
		6	183.0 ± 28.2	222.0 ± 23.6	70.7 ± 12.1	47.6 ± 0.4	38.4 ± 1.4	34.7 ± 0.29	160.3 ± 39.0
		8	181.0 ± 27.5	147.3 ± 29.7	68.5 ± 18.7	38.1 ± 1.1	29.3 ± 2.6	23.0 ± 3.0	127.0 ± 26.9
		24	155.0 ± 24.5	99.9 ± 0.2	34.9 ± 4.5	23.4 ± 5.2	18.7 ± 1.8	12.5 ± 1.5	124.3 ± 29.9
3	48	0	290.7 ± 37.4	330.0 ± 35.8	337.7 ± 33.3	435.3 ± 55.5	517.7 ± 58.5	543.7 ± 40.9	101.5 ± 19.9
		2	236.67 ± 16.7	88.6 ± 11.3	90.9 ± 19.1	79.3 ± 34.4	102.9 ± 11.5	85.2 ± 13.9	110.7 ± 10.1
		4	159.7 ± 25.9	62.0 ± 8.3	47.8 ± 14.3	49.4 ± 4.0	47.5 ± 8.0	49.7 ± 9.3	101.3 ± 1.5
		6	74.5 ± 21.1	44.4 ± 8.4	40.4 ± 2.7	30.0 ± 3.7	46.0 ± 13.4	35.4 ± 1.5	104.8 ± 9.9
		8	49.1 ± 16.4	29.1 ± 9.9	26.3 ± 8.0	25.0 ± 1.7	32.3 ± 8.8	30.0 ± 3.0	102.4 ± 5.6
		24	41.3 ± 1.6	19.8 ± 5.8	12.7 ± 2.2	15.9 ± 6.7	17.2 ± 4.4	18.6 ± 10.8	101.0 ± 4.6
4	57	0	457.7 ± 40.2	498.3 ± 34.3	536.7 ± 55.9	634.7 ± 37.2	760.0 ± 23.9	826.0 ± 61.6	114.0 ± 16.6
		2	136.0 ± 7.9	105.8 ± 11.6	99.4 ± 5.5	97.0 ± 15.1	90.3 ± 9.5	90.6 ± 2.5	118.7 ± 3.2
		4	81.0 ± 7.9	66.5 ± 3.3	75.2 ± 13.1	69.3 ± 11.8	79.7 ± 13.8	85.8 ± 2.3	101.7 ± 7.6
		6	59.4 ± 0.6	58.2 ± 3.3	46.8 ± 2.2	47.1 ± 1.8	43.3 ± 2.9	38.3 ± 2.9	110.0 ± 10.0
		8	48.5 ± 2.0	37.0 ± 4.0	30.9 ± 0.9	35.2 ± 4.6	31.3 ± 5.5	31.0 ± 1.7	106.7 ± 5.8
		24	23.3 ± 7.5	21.9 ± 1.9	18.8 ± 1.2	15.8 ± 2.7	18.2 ± 1.7	19.6 ± 2.5	76.2 ± 7.7
5	71	0	339.7 ± 10.0	533.3 ± 41.6	506.3 ± 11.85	602.0 ± 13.1	623.3 ± 25.2	613.3 ± 18.6	144.0 ± 30.4
		2	263.7 ± 23.7	110.3 ± 11.9	79.2 ± 4.6	90.3 ± 3.2	102.2 ± 6.9	126.0 ± 4.6	119.0 ± 14.5
		4	83.7 ± 3.3	65.1 ± 3.2	47.9 ± 4.6	62.6 ± 6.2	59.0 ± 8.1	55.0 ± 8.7	105.7 ± 12.5
		6	84.3 ± 4.0	44.6 ± 5.9	35.4 ± 2.4	44.5 ± 4.0	45.6 ± 13.2	57.8 ± 0.4	123.0 ± 21.1
		8	91.7 ± 7.6	41.8 ± 2.2	29.8 ± 6.9	36.0 ± 4.2	40.0 ± 7.9	45.4 ± 11.3	116.7 ± 14.6
		24	39.6 ± 15.9	19.6 ± 2.1	12.9 ± 1.5	12.9 ± 1.5	14.7 ± 1.3	15.1 ± 3.1	111.7 ± 15.5
6	219	0	260.3 ± 11.7	331.7 ± 17.2	423.3 ± 25.2	495.0 ± 59.1	585.0 ± 43.9	657.3 ± 30.6	128.0 ± 19.2
		2	214.3 ± 16.2	101.9 ± 15.0	81.7 ± 11.7	77.4 ± 9.8	82.2 ± 8.1	96.0 ± 3.0	155.3 ± 34.0
		4	82.7 ± 8.3	66.5 ± 19.2	50.5 ± 8.1	47.6 ± 1.9	57.9 ± 6.4	74.0 ± 4.9	114.6 ± 21.6
		6	124.7 ± 70.5	61.8 ± 5.5	44.0 ± 2.6	40.4 ± 7.8	43.7 ± 7.0	59.5 ± 13.0	112.5 ± 17.7
		8	69.8 ± 10.4	46.5 ± 6.0	29.0 ± 3.4	31.0 ± 1.7	32.2 ± 2.8	44.1 ± 4.4	107.5 ± 21.0
		24	34.9 ± 10.9	19.2 ± 5.5	12.0 ± 2.2	10.2 ± 3.6	13.9 ± 1.0	12.6 ± 3.7	117.0 ± 20.7
7	168	0	289.3 ± 7.0	339.3 ± 22.5	429.7 ± 20.0	505.3 ± 26.8	595.7 ± 6.7	645.3 ± 41.3	219.3 ± 10.8
		2	210.0 ± 13.1	99.55 ± 8.0	82.4 ± 10.8	83.1 ± 16.4	86.5 ± 13.8	82.5 ± 2.3	115.7 ± 9.6
		4	76.2 ± 18.1	65.8 ± 14.7	53.2 ± 11.9	54.3 ± 12.8	60.1 ± 4.8	57.4 ± 10.9	110.3 ± 6.4
		6	69.8 ± 7.6	44.7 ± 2.5	42.7 ± 9.1	42.8 ± 8.3	46.4 ± 4.3	54.3 ± 16.3	101.3 ± 4.2
		8	58.0 ± 13.9	34.8 ± 0.8	32.2 ± 5.4	28.7 ± 2.2	34.4 ± 9.6	36.0 ± 13.5	111.0 ± 15.8
		24	30.6 ± 12.5	17.0 ± 0.8	17.5 ± 3.5	16.7 ± 1.6	14.9 ± 1.5	15.8 ± 3.8	99.0 ± 22.3

Based on perceptions of the chemical nature of flocculation processes, it is logical that the higher the content of the flocculant, the more intensive flocculation is, and this was observed during the tests.

Tests of polysept of different batches (at the concentration of 0.007%) for its ability to precipitate cell debris showed the following: after 4 hours, four of the tested batches demonstrated a 68.3–82.0% (by 3.1–5.7 times) decrease in turbidity, which declined to medium values (the differences are significant, $p < 0.005$); batch No. 48 demonstrated a 74% (by 3.9 times) decrease in turbidity after 6 hours; batch No. 343 demonstrated an 88% (by 8.2 times) decrease in turbidity after 24 hours; batch No. 522 demonstrated no cell debris precipitation even after 24 hours (Fig. 2A, Table 2).

When the amount of the added flocculant was increased by 1.5 times (0.0105%), six of the tested batches demonstrated a 57–81% (by 2.3–5.3 times) decrease in turbidity of the polymer-supplemented suspension; however, the turbidity still remained rather high (Fig. 2B, Table 2). After 4 hours, five PHMG hydrochloride batches demonstrated an 80–87% decrease in turbidity, which declined to medium values (16–100 FNU). Batch No. 343 demonstrated a decrease in turbidity after 6 hours, and batch No. 522 did not demonstrate satisfactory cell debris precipitation even after 24 hours.

In further tests, when polysept concentration was increased to 0.05316% (by 7.5 times), rapid precipitation of debris was observed as soon as after 2 hours. After 24 hours (according to FMD vaccine production technology), the turbidity of the suspension was 10–20 FNU, and this was indicative of satisfactory flocculation (Fig. 2C–F, Table 2).

Debris sedimentation was observed in the control samples containing no flocculant (Fig. 2G, Table 2); however, turbidity decreased only by 1.1–2.2 times as a result of natural sedimentation, and, after 24 hours, the turbidity still exceeded 100 FNU, being unsatisfactory in terms of the vaccine production technology.

At the following stages of the study, the concentrations of total viral protein and FMDV antigen composition were determined.

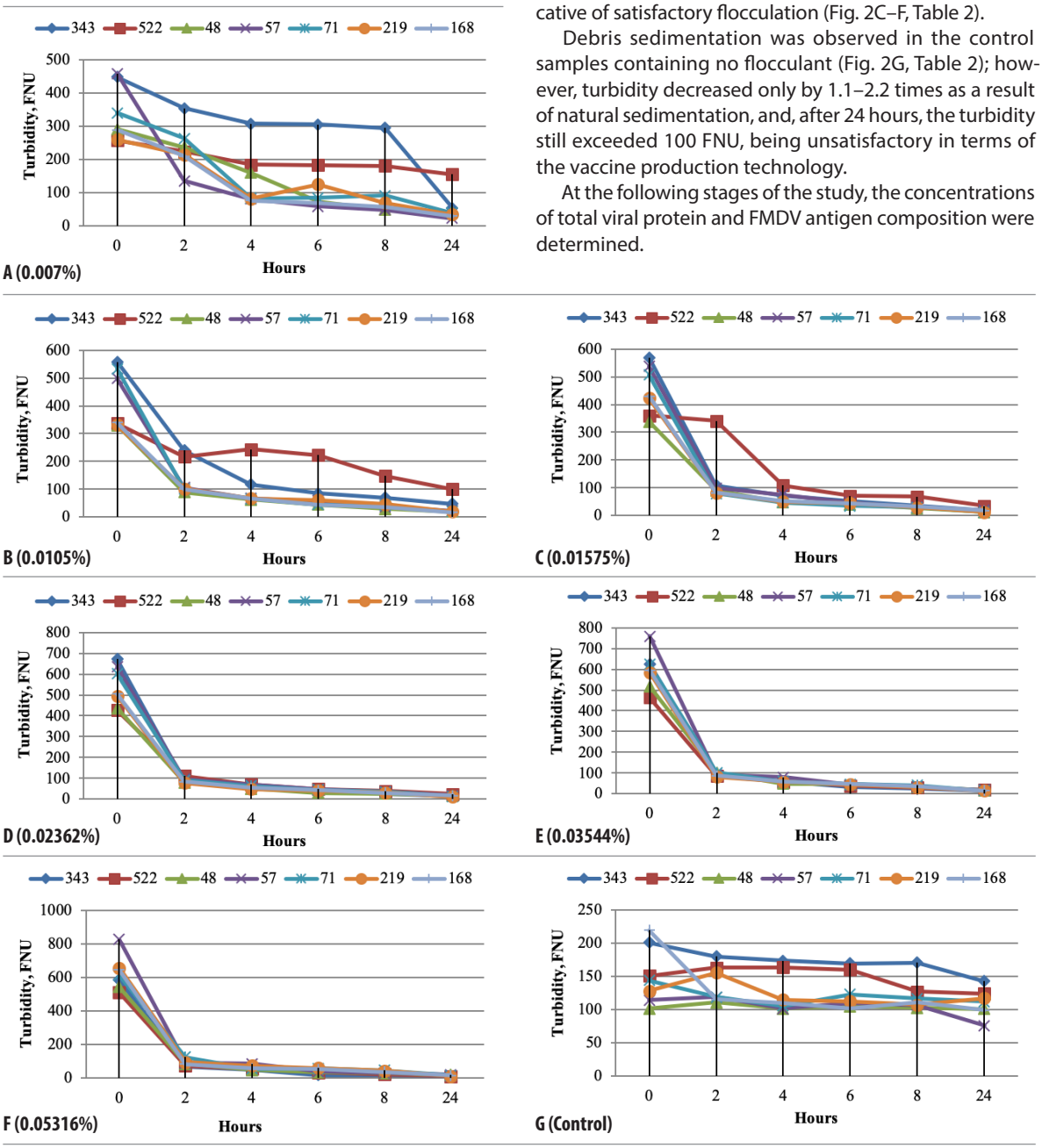

Fig. 2. Dynamics of flocculation properties of polysept batches at different polymer concentrations

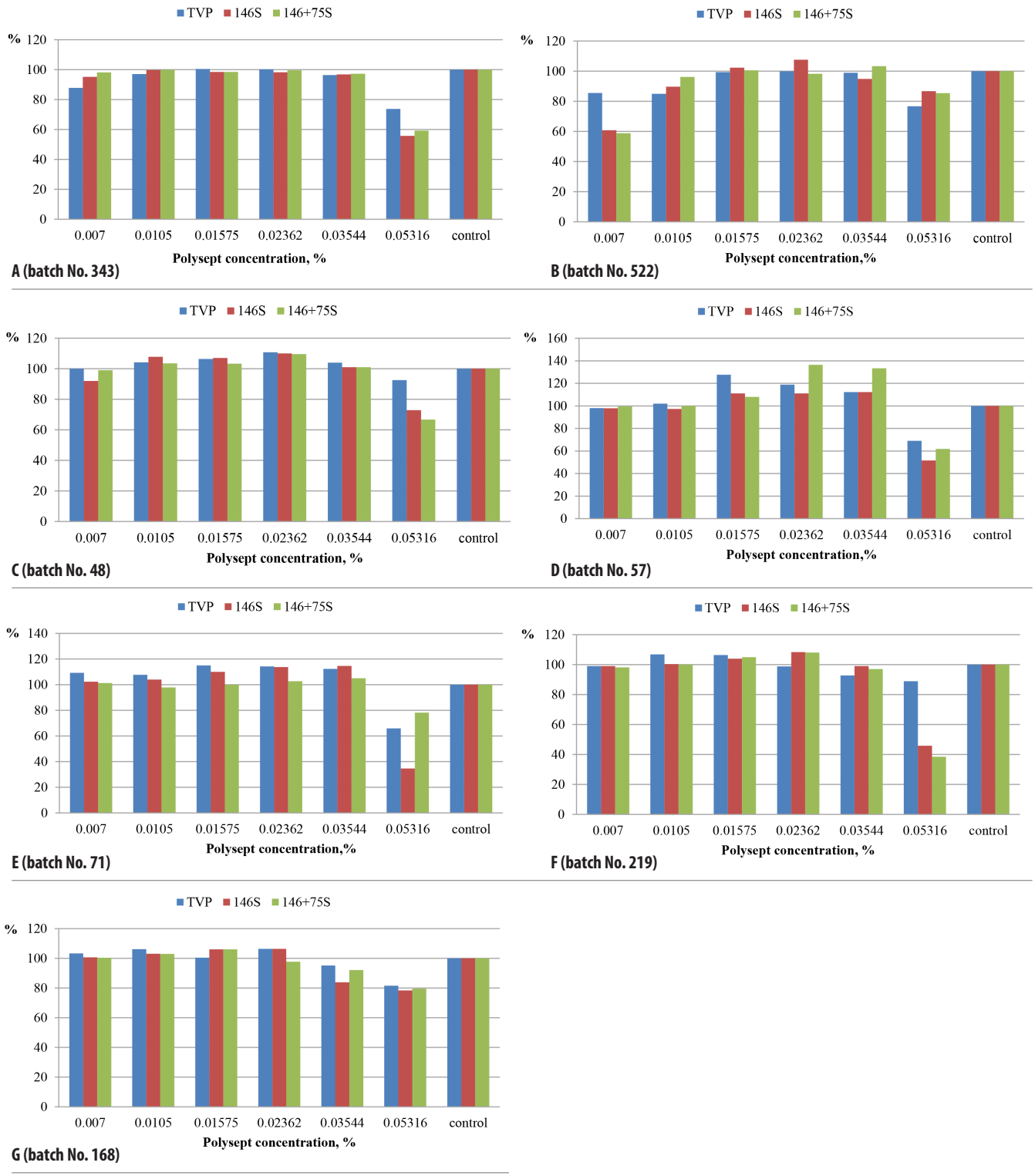

Fig. 3. Relative changes in concentrations of total viral protein (TVP) and immunogenic components of FMD virus supplemented with polysept of different batches

The study involved the use of antigens of different FMDV strains, and it was therefore incorrect to apply absolute measures when calculating losses at different PHMG hydrochloride concentrations. In view of this, the losses were reported using relative measures, with measures in the control production sample (0.01% of polysept) taken as 100%. The test results are presented in Figure 3.

Since the use of higher flocculant concentrations resulted in better antigen purification and lower anticomplementary activity of viral antigen preparations as determined with CFT, this could probably explain a slight rise in the content of total viral protein and immunogenic components of FMD virus when polysept concentration was increased from 0.007 to 0.03544%.

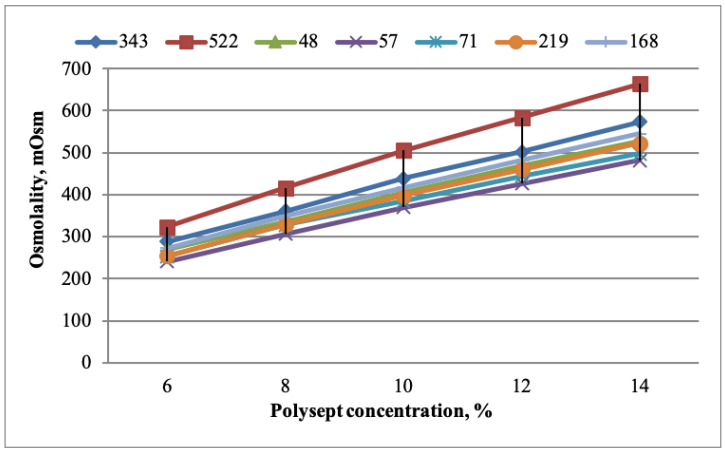

Fig. 4. Osmolality dynamics at different polysept concentrations

Table 3
Osmolality reference values for determination of polyhexamethylene guanidine hydrochloride suitability for FMD vaccine production

PHMG hydrochloride concentration, %	Osmolality, mOsm
6	260 ± 20
8	330 ± 25
10	400 ± 25
12	460 ± 30
14	520 ± 20

All the batches of polysept at the concentration of 0.05316% demonstrated an 8.0–65.4% decrease in FMDV total viral protein and immunogenic component content. It was found that, when the turbidity of the suspension was very high, CFT tests yielded false (erroneously low) results, and the immunogenic component content was 14.5–39.3% lower as compared with the control (Fig. 3).

At the final stage, the osmolality of solutions of polysept of all the tested batches was measured at different concentrations of the flocculant (Fig. 4). Batch No. 522 polysept osmolality was considerably different from that of other batches at all the tested concentrations (the differences were significant, $p < 0.001$). In particular, at PHMG hydrochloride concentration of 6%, the osmolality of this batch was 324 ± 4 mOsm, whereas in other batches it varied from 241 ± 3 to 288 ± 4 mOsm. As for 14% polymer solutions, the differences were even higher: 664 ± 8 mOsm in batch No. 522, and 482 ± 5 and 573 ± 10 mOsm in the rest of the batches.

Thus, to determine the suitability of polysept batches for FMD vaccine production, it is necessary to test the polymer for its flocculation properties at different concentrations (0.007, 0.0105, 0.01575%) over the period of 24 hours. The application of higher PHMG hydrochloride concentrations is economically disadvantageous.

The production of FMD vaccines involves the use of a 10% polysept solution. At this PHMG hydrochloride concentration, all the batches found suitable for production demonstrated the osmolality values that ranged from 370 mOsm to 440 mOsm. Batch No. 522 demonstrated a higher osmolality, namely 504 ± 5 mOsm.

CONCLUSION

As a result of the tests performed, the following indicators of PHMG hydrochloride suitability for FMD vaccine production were determined:

– flocculation properties of a batch of polysept at different concentrations (0.0105 and 0.01575%) evaluated in dynamics over 24 hours. After this period, the turbidity of 0.0105 and 0.01575% polysept solutions should not exceed 30 FNU (Table 2);

– osmolality of PHMG hydrochloride solutions measured at different concentrations (6, 8, 10, 12, 14%). The osmolality should be within the reference values specified in Table 3.

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