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Evaluation of precipitation time of the aluminum salts adsorbed potentially frozen vaccines used in the Polish National Immunization Schedule for their pre-qualification before the administration

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Purpose: Vaccines adsorbed on aluminum adjuvants irreversibly lose potency after freezing and their safety is affected. To prevent the administration of such vaccines, the World Health Organization developed the Shake Test designed to determine whether adsorbed vaccines have been frozen or not. However, the Shake Test is difficult and time-consuming when routinely conducted at the place of vaccination. In this study, a modified shake test for prequalification of potentially frozen vaccines was elaborated.

Materials and Methods: Vaccines used in the Polish Immunization Schedule were investigated and the analysis includes an assessment of precipitation time and the influence of the container type, amount and type of aluminum compound, and a volume of vaccine dose on the precipitation time.

Results: Significant differences between the precipitation time of frozen and non-frozen vaccines routinely used in the Polish Immunization Schedule were observed. The precipitation time of all non-frozen vaccines was above 30 minutes. The longest precipitation time of frozen vaccines was 10 minutes.

Conclusion: The finding of the study can be used in practice by the personnel administering vaccines to patients. Step-by-step recommendations for the preparation of the test have been proposed in the article.

Keywords: Vaccines, Cold chain, Shake test, Efficacy

Introduction

Appropriate storage of vaccines is crucial for the retention of their efficacy and safety. Both European Pharmacopoeia and the World Health Organization (WHO) recommend that the storage temperature of vaccines for human use is 5°C±3°C, and liquid adsorbed vaccines must not be allowed to freeze [1-4]. Accidental freezing of aluminum-based vaccines relatively often happens during their storage and transportation, in both developing and developed countries, the problem exists for a long time and many literature data indicate the strong need to improve vaccines' storage conditions [5-7]. Vaccines exposed to the freezing temperatures irrecoverably lose their potency, the structure of aluminum adjuvant is destroyed, and adsorbed antigen is detached. Damages that affect the freeze-sensitive aluminum adjuvanted vaccines after freezing

develop through the separation of lattices between the aluminum adjuvant and the antigen. This causes formation of aluminum aggregates, and inadvertent loss of vaccine's potency. Freezing of vaccines not only can result in compromised immunogenicity but also influences their safety, causing sterile abscesses in the injection site [8,9].

To prevent the administration of vaccines that may potentially be not effective and safe after accidental freezing, WHO developed the Shake Test, designed to determine whether adsorbed vaccines have been affected by freezing or not. This test was validated in 2010 by the study team organized and supervised by WHO with the result of a 100% positive predictive value [10-12].

In the vaccination points however routine performing the shake test is difficult and time-consuming, as it requires simultaneous comparison of the precipitation time of the vaccine to be administered with the same kind and batch of the vaccine that was solid frozen [10-12]. Moreover, the Shake Test requires that an additional dose of vaccine, as a frozen control must be used, which may be of importance in case a given vaccine is lacking on the market. Performing this test before vaccination also poses a risk that by mistake the frozen control vaccine will be administered.

The aim of the study was to determine the precipitation time that would allow a distinction between a frozen and non-frozen vaccine without having to be compared to a frozen control, which could simplify testing at vaccination points. The possible influence of a container type, volume of a dose, and amount of aluminum in one dose on the results of the test were also verified.

Materials and Methods

Vaccines

In the study, 15 types of vaccines produced by five manufacturers were used (Table 1). The vaccines were packed in three types of containers: pre-filled syringes, vials, and ampoules. The volume of one dose was either 0.5 mL or 1 mL. For most of the vaccines, aluminum hydroxide was used as an adjuvant. Only three vaccines were adsorbed on aluminum phosphate. The amount of aluminum given in specifications for a given vaccine varied from 0.1 to 1.25 mg in one dose.

Vaccines were stored at the recommended temperature of 5°C±3°C, in the laboratory refrigerator POL-EKO CHS 700 A with the temperature monitoring system (software EasyLab Professional). Frozen vaccines were kept at a temperature of approximately -25°C, in the freezer (Bosch duo-system), monitored daily with the calibrated thermometer.

Estimation of freezing time optimal for the study

In order to establish the same and uniform freezing time of the vaccines used in the study, the following test was performed. Vaccines against diphtheria, tetanus, and pertussis (DTP, IBSS BIOMED S.A.) of the same batch, as a representative matrix for all the vaccines used in the study, were divided into five groups, each containing 6 ampoules and put in the freezer, where the temperature was approximately -25°C. The 1st group of vaccines was exposed to freezing temperature for 1 hour. The 2nd group of vaccines was exposed to freezing temperature for 2 hours. The 3rd group of vaccines was exposed to freezing temperature for 3 hours. The 4th group of vaccines was exposed to freezing temperature for 6 hours, and the 5th group of vaccines was exposed to freezing temperature for 24 hours.

Assessment of vaccines' precipitation time

The assessment of vaccines' precipitation time was performed on the basis of the Shake Test procedure, described in the "Bulletin of the World Health Organization" [10]: In a typical demonstration of the shake test, two identical vials of a vaccine (i.e., from the same batch and the same manufacturer) that is suspected of having been exposed to freezing temperatures are selected; one of the two vials is purposely frozen and then thawed as the negative control, while the second vial serves as the vial to be "tested" against this negative control. The two vials are held together in one hand and vigorously shaken for 10 seconds; they are then placed side by side on a flat surface. Provided the test vial has not been frozen, sedimentation is slower in the test vial than in the control vial that has been frozen and thawed. If the test vial has been frozen, the test and control vials will have similar sedimentation rates.

Visual examination

Visual examination of precipitation in frozen and non-frozen vaccines was evaluated by the WHO trained personnel, as described above, with the use of the Adelphi Apollo apparatus, according to the requirements of European Pharmacopoeia 2.9.20. "Particulate contamination: visible particles". This apparatus consists of a viewing station that comprises a matt black panel of appropriate size held in a vertical position, a non-glare white panel of appropriate size held in a vertical position next to the black panel, and an adjustable lamp hold-

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Table 1. Vaccines used in the study

Trade name	Type of vaccine	Manufacturer	Type of the container	Type of the aluminum adjuvant	Amount of aluminum in one dose (mg)	One dose volume (mL)
Adacel	Vaccine against diphteria, tetanus and pertussis	Sanofi Pasteur	Pre-filled syringe	Aluminum hydroxide	0.30–0.36	0.5
Adacel	Vaccine against diphteria, tetanus and pertussis	Sanofi Pasteur	Vial	Aluminum hydroxide	0.60–0.72	1
Boostrix	Vaccine against diphteria, tetanus and pertussis	GlaxoSmithKline	Pre-filled syringe	Aluminum hydroxide	0.4–0.6	0.5
DT	Vaccine against diphtheria and tetanus	IBSS BIOMED S.A.	Ampoule	Aluminum hydroxide	Max. 0.7	0.5
DTP	Vaccine against diphteria, tetanus and pertussis	IBSS BIOMED S.A.	Ampoule	Aluminum hydroxide	Max. 0.7	0.5
Engerix B	Vaccine against hepatitis B	GlaxoSmithKline	Pre-filled syringe	Aluminum hydroxide	0.175–0.325	0.5
Engerix B	Vaccine against hepatitis B	GlaxoSmithKline	Vial	Aluminum hydroxide	0.35–0.65	1
Euvax B	Vaccine against hepatitis B	LG Life Sciences Poland Sp. z o. o.	Vial	Aluminum hydroxide	Max. 2.50	0.5
Euvax B	Vaccine against hepatitis B	LG Life Sciences Poland Sp. z o. o.	Vial	Aluminum hydroxide	Max. 1.25	1
Hexacima	Vaccine against diphteria, tetanus, pertussis, hepatitis B, *Haemophilus influenzae* type b and poliomyelitis	Sanofi Pasteur	Pre-filled syringe	Aluminum phosphate	0.50–0.70	0.5
Infanrix Hexa	Vaccine against diphteria, tetanus, pertussis, hepatitis B, *Haemophilus influenzae* type b and poliomyelitis	GlaxoSmithKline	Pre-filled syringe	Aluminum phosphate	0.55–0.85	0.5
Infanrix IPV-Hib	Vaccine against diphteria, tetanus, pertussis, *Haemophilus influenzae* type b and poliomyelitis	GlaxoSmithKline	Pre-filled syringe	Aluminum hydroxide	0.40–0.60	0.5
Pentaxim	Vaccine against diphteria, tetanus, pertussis, *Haemophilus influenzae* type b and poliomyelitis	Sanofi Pasteur	Pre-filled syringe	Aluminum hydroxide	0.20–0.45	0.5
Prevenar	Vaccine against *Streptococcus pneumoniae*	Wyeth LLC	Pre-filled syringe	Aluminum hydroxide	0.1–0.15	0.5
Synflorix	Vaccine against *Streptococcus pneumoniae*	GlaxoSmithKline	Pre-filled syringe	Aluminum hydroxide	0.40–0.60	0.5

Manufacturers warn against freezing aluminum adsorbed vaccines in product information documents (summary of product characteristics, package leaflet, and labeling).
DTP, diphtheria, tetanus, and pertussis.

er fitted with a suitable, shaded, white-light source and with a suitable light diffuser (a viewing illuminator containing two 13 W fluorescent tubes, each 525 mm in length). The intensity of illumination at the viewing point is maintained between 2,000 lux and 3,750 lux. For the assessment of precipitation time of frozen and non-frozen vaccines the certified, calibrated mechanical chronometer was used.

Statistical analysis

The arithmetic mean, median, and standard deviations were calculated using Excel (Microsoft Corp., Redmond, WA, USA). Statistical analysis was performed using the Kruskal-Wallis test, which is suitable for comparing two or more independent samples of the same or different sizes. The results were regarded as significant at a p-value of <0.01. The false-discovery rate (FDR) was calculated based on p-values. The sensitivity and specificity of the test were calculated as follows: $A/(A+C)\times100\%$ and $D/(B+D)\times100\%$, respectively, where A is the number of frozen vaccine samples precipitating for ≤10 minutes, B is the number of non-frozen vaccine samples precipitating for ≤10 minutes, C is the number of frozen vaccine samples precipitating for >10 minutes, and D is the number of non-frozen vaccine samples precipitating for >10 minutes.

Results

In the preliminary experiments concerning estimation of freezing time optimal for the study, it occurred that the time of sedi-

mentation of conglomerates of adsorbent which was detached from the antigen as a result of freezing is related to freezing time. In Table 2, the precipitation time of the DTP vaccine depending on freezing time is presented.

Based on the observation that the significant differences in the precipitation time of the vaccine exposed to freezing temperatures occurred solely in the first freezing phase (1–2 hours) and after 3 hours of freezing the precipitation time stabilizes and is quite similar (90–120 seconds) we decided to apply the freezing time of 6 hours for the investigation of all the vaccines in the study.

Precipitation time of the frozen vaccines was compared to the same vaccines of the same batch stored at the recommended temperature of 2°C–8°C. In Table 3 the precipitation time of tested vaccines exposed to freezing temperature (-25°C), taking to account the type of the primary packaging, is presented together with the calculated mean and standard deviation. Statistically significant differences in precipitation time were observed among vaccines packed in different types of containers (p-value=0.00001 calculated for comparison of all three types of containers, FDR is significant).

The sensitivity and specificity of the precipitation test for all examined vaccine samples were calculated as 100% (confidence interval=95%). Table 3 contains data on the precipitation time of vaccines exposed for 6 hours to freezing temperature (-25°C), and Table 4 presents the results of the test for all investigated samples.

The longest precipitation time was observed for vaccines packed in vials, followed by vaccines packed in pre-filled syringes (Fig. 1).

The fastest precipitation occurred in ampoules. To verify whether the precipitation time was related to the volume of a vaccine dose, we compared three vaccines (Adacel, Engerix B, and Euvax B) each available in doses in two volumes: 0.5 mL and 1 mL. The analysis revealed no significant differences

Table 2. Precipitation time of DTP vaccine (6 vials per each freezing time group) exposed to freezing temperature (-25°C), dependently on the time of exposure

Freezing time (hr)	Precipitation time (sec)
1	600
2	270
3	120
6	90
24	90

DTP, diphtheria, tetanus, and pertussis.

Table 4. The two-by-two table indicating the value of the diagnostic test described in this paper

	Frozen vaccines	Non-frozen vaccines
Precipitation time ≤10 min	90	0
Precipitation time >10 min	0	90

Table 3. Precipitation time of vaccines exposed for 6 hours to freezing temperature (-25°C).

Type of the container	Vaccine	One dose volume (mL)	Sample						Mean±SD
			1	2	3	4	5	6	
Pre-filled syringe	Adacel	0.5	230	230	260	230	250	240	240±12.64911
	Boostrix	0.5	86	85	95	90	90	95	90±4.262237
	Engerix B	0.5	90	85	95	85	100	85	90±6.324555
	Hexacima	0.5	275	270	270	265	268	272	270±3.405877
	Infanrix Hexa	0.5	135	140	139	142	143	141	140±2.828427
	Infanrix IPV-Hib	0.5	92	88	90	89	90	91	90±1.414214
	Pentaxim	0.5	76	80	70	77	74	73	75±3.464102
	Prevenar	0.5	80	75	74	71	73	79	75±3.50238
	Synflorix	0.5	110	100	105	105	90	90	100±8.3666
Vial	Adacel	1	630	630	595	580	580	585	600±23.87467
	Engerix B	1	330	340	342	330	320	320	330±9.416298
	Euvax B	0.5	295	305	310	305	295	295	300±6.645801
	Euvax B	1	240	240	240	245	230	240	240±4.91596
Ampoule	DT	0.5	93	90	95	85	88	90	90±3.544949
	DTP	0.5	80	80	95	95	95	95	90±7.745967

SD, standard deviation; DT, diphtheria and tetanus; DTP, diphtheria, tetanus, and pertussis.

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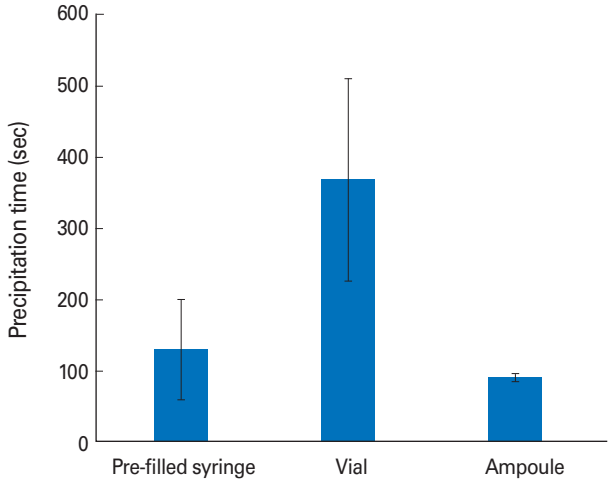

Fig. 1. Precipitation time for vaccines packed in different types of containers.

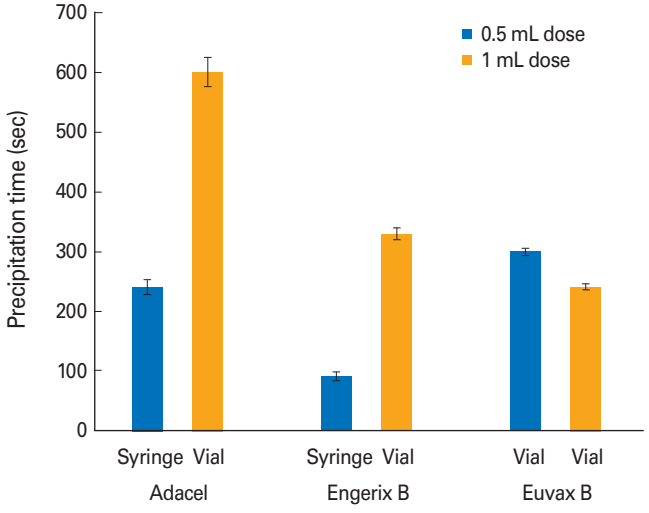

Fig. 2. Differences in precipitation time dependently on the container type.

(p-value=0.98231, FDR is not significant) when all three vaccines were analyzed. However, different doses of the two vaccines: Adacel and Engerix B were packed in different containers: 0.5 mL dose in a pre-filled syringe and 1 mL dose in vial (Fig. 2). When we compared the precipitation time between Adacel and Engerix B packed in similar containers (the same dose), we revealed significant differences (p-value=0.00395 for both pre-filled syringe and vials, FDR is significant).

Only in the case of Euvax B, both doses were packed in the same type of container, which was a vial. Statistical analysis showed significant differences in precipitation time between 0.5 mL dose and 1 mL dose of Euvax B vaccine (p-value=0.00395, FDR is significant).

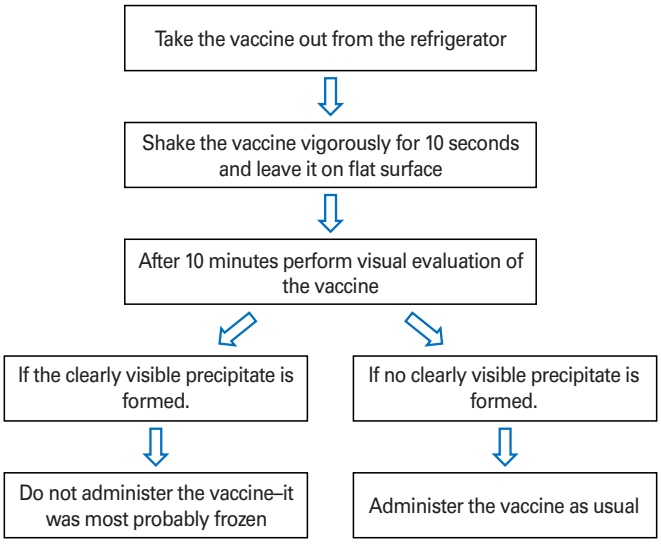

Fig. 3. Recommendation for the test verifying if the vaccine was frozen.

No correlation was observed between the type of the adjuvant (aluminum hydroxide versus aluminum phosphate) and the precipitation time. We were not able to assess the correlation between approved aluminum adjuvant specifications and vaccines' precipitation time because the concentration range of aluminum adjuvant varied strongly among tested vaccines.

Fig. 3 presents recommendations for the preparation of the test that could be performed by the personnel administering vaccines to patients to distinguish frozen vaccines from non-frozen ones and Fig. 4 shows examples of the test results for vaccines packed in different types of containers.

Discussion

Aluminum adsorbed vaccines exposed to freezing temperatures lose their physical, chemical, and immunological properties and their administration can result not only in compromised immunogenicity but also increase adverse local reactions at an injection site, such as sterile abscesses [7-9]. Inadvertent vaccines' freezing still happens often worldwide, although many efforts are being undertaken to prevent the patients from getting vaccinated with ineffective and unsafe products. These efforts aim in three directions: (1) improvement of the cold chain consistency and reliability; (2) enhancing knowledge concerning the mechanisms of frozen vaccines destruction and attempts to improve their temperature stability; and (3) implementation of methods that allow distinguishing non-frozen and frozen vaccines.

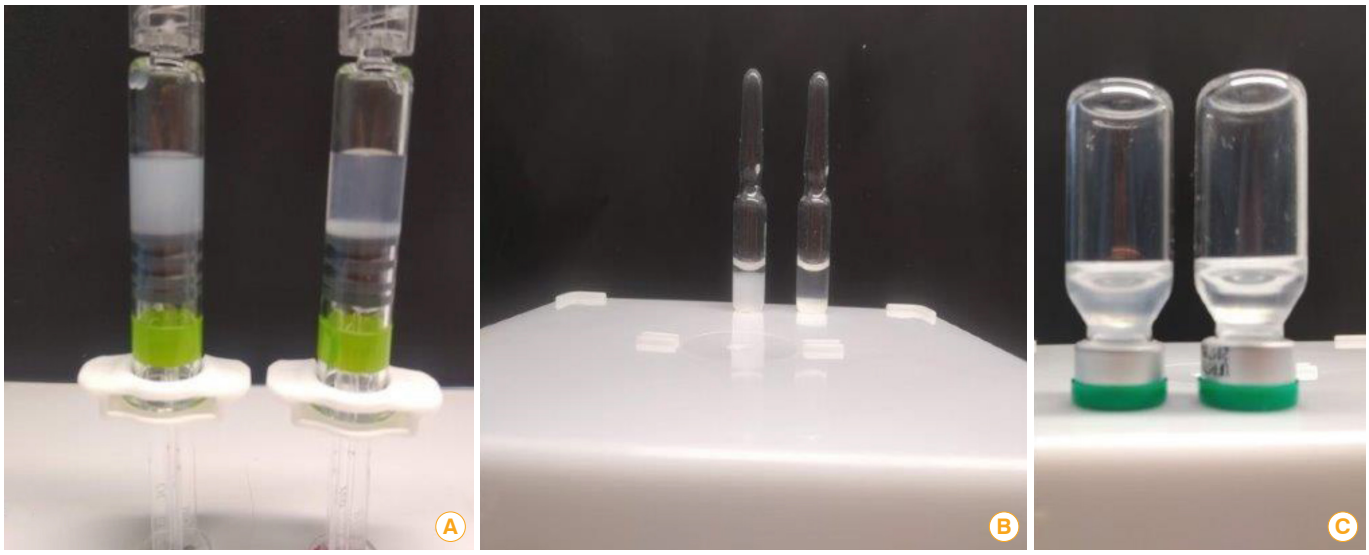

Fig. 4. Example of non-frozen (left side) and frozen (right side) vaccines packed in the different types of containers after 10 minutes from shaking for 10 seconds. (A) The pre-filled syringe. (B) The ampoule. (C) The vial.

Many efforts are being undertaken worldwide to improve the functionality of the cold chain, as accidental freezing of vaccines is not only dangerous for the patients but also causes major budgetary losses. These efforts aim at three main goals: improvement of systems to monitor the temperature of thermo-sensitive vaccines, implementation of appropriate equipment to store and transport vaccines, and providing a sufficient number of adequately trained staff to handle vaccines [13-20].

Literature data indicate that the loss of potency in frozen vaccines is attributed to the aggregation of adjuvant particles. After freezing, the lattice (made up of bonds between the adsorbent and the antigen) in a vaccine is broken. Separated adsorbent tends to form larger, heavier granules that gradually settle at the bottom of the vial. Sophisticated studies performed with the use of many different methods indicated significant physical and chemical differences between the freeze-damaged and non-frozen vaccine adjuvant and proved that the mechanisms of the frozen vaccine destruction are very complicated. They involve among others detachment of water from aluminum hydroxide hydrate particles that leads to the crushing of the viscous Alhydrogel structure [8,9]. The studies currently focus on resolving this problem aim at recognizing the mechanisms of frozen vaccines' destruction and improvement of their temperature stability addition of compounds that stabilize aluminum adsorbed proteins in solutions, including osmolytes and cryoprotectants, such as glycine, propylene glycol, PEG 300, sucrose, and trehalose [21-28].

In this study, we were not able to find the correlation between the approved aluminum adjuvant specifications range and vaccines' precipitation time. However, unpublished data indicate that a lower concentration of aluminum in the vaccine prolongs the precipitation time after freezing, thus making it difficult to assess performing the Shake Test whether the product has been frozen. Analysis by scanning electron microscopy, phase contrast microscopy, and energy dispersive spectroscopy (EDS) confirmed the above results. The percentages by weight (Wa%) and atomic percent (AT%) data for aluminum from EDS spectra were compared in terms of "impossible" and "possible" samples to be distinguished in the Shake Test. It was shown that the weight percent and atomic percentage of aluminum in vaccine groups where the Shake Test was impossible to perform is approximately 50% lower than in groups where a distinction between frozen and non-frozen samples was possible with this test [29] (prof. Wiesław Kurzątkowski personal communication).

In order to enable distinguishing between frozen and non-frozen vaccines before administration, the Shake Test was designed by a study team in WHO in 2010. The Shake Test is based on the difference in sedimentation time between frozen and non-frozen aluminum adsorbed vaccines. In the container with the tested vaccine which has not been frozen, sedimentation is slower than in the container with the positive control vaccine that has been frozen and thawed. In case the tested vaccine was frozen, sedimentation time in both tested and control vaccines' containers is similar [10-12].

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However, in practice, the Shake Test is rarely or never performed on a routine basis prior to the vaccination. The most probable reasons are as follows: (1) the Shake Test requires special preparations prior it is possible to perform (freezing to the solid state the same kind and a batch of the tested vaccine); (2) the test is time-consuming and requires personnel training; (3) there's not enough awareness of the threat of administration of frozen vaccines; and (4) there is no awareness of the Shake Test existence.

Therefore, this study focused on finding an easier and quicker alternative to the Shake Test, which would not require the control sample and special training. Studies were based on the results presented by Kurzątkowski et al. [8] and Kartoglu et al. [10]. The test developed in the present study is easy to perform and does not require additional time. (It can be performed while the vaccine is left to achieve room temperature prior to administration.) It is intended to reduce the risk of administration of a vaccine that has been accidentally frozen.

In the study, it was demonstrated that the precipitation time of all non-frozen vaccines investigated was above 30 minutes whereas the longest precipitation time of frozen vaccines was 10 minutes. If the product precipitation time is less than/equal to 10 minutes, additional studies (Shake Test) should be performed to confirm if the vaccine was frozen or not. Vaccines in vials manifest less visible precipitation than in pre-filled syringes or ampoules and therefore the precipitation time necessary for reading the results of the test was longer. When the same type of container was used the precipitation was less visible in the smaller volume of the vaccine. No correlation was revealed between the type of the adjuvant (aluminum hydroxide versus aluminum phosphate) and the precipitation time.

In conclusion, in this study precipitation time of frozen and non-frozen vaccines used in the Polish Immunization Schedule was measured and compared. Obtained data indicate that there is a significant difference between the precipitation time of frozen and non-frozen vaccines. This finding can be used in practice by the personnel administering vaccines to patients.

It has been proven that if the significant precipitation of the vaccine to be administered occurs (after shaking for 10 seconds) 10 minutes before immunization, this would suggest that the product might be exposed to freezing temperature and should not be administered. The precipitation time of non-frozen vaccines was always longer than 30 minutes.

This vaccine batch should also be further examined in the specialized laboratory on the possibility of being accidentally frozen. The proposed evaluation of the precipitation time of the product before vaccination is much quicker and easier than using the Shake Test. Regardless of the precipitation evaluation, each vaccine should be taken out of the refrigerator to achieve the room temperature before injection; therefore, no additional time would be needed. The person administering the vaccine should only evaluate the appearance of the vaccine before its administration and check if the precipitate is formed.

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