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Structural damages in adsorbed vaccines affected by freezing

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ABSTRACT

This study was planned to evaluate structural damages in adsorbed vaccines affected by freezing using scanning electron microscopy and X-ray analysis of the elements. Randomly selected 42 vials of eight different types of WHO pre-qualified adsorbed freeze-sensitive vaccines from 10 manufacturers were included in the study. Vaccines were kept at 5 °C. Selected numbers of vials from each type were then exposed to −25 °C for 24 h periods. All samples were evaluated for their structure using scanning electron microscopy, X-ray analysis of the elements and precipitation time. Scanning electron microscopy of vaccines affected by freezing showed either smooth or rough surfaced conglomerates associated with phosphate content of the precipitate. These vaccines precipitated 2–15 times faster compared to non-frozen samples. Non-frozen samples showed uniform flocculent structure either dense or dispersed. X-ray analysis of precipitates in frozen samples confirmed that the precipitate is mainly aluminium clutters. Scanning electron microscopy confirmed that the lattice structure of bonds between adsorbent and the antigen is broken and aluminium forms conglomerates that grow in size and weight. The precipitation time of vaccines affected by freezing is 4.5 times faster on average compared to non-frozen samples. These facts form the basis of the "shake test".

1. Introduction

In order to ensure potency and safety of liquid formulation of aluminium-based vaccines containing diphtheria, pertussis, tetanus, hepatitis B, *Haemophilus influenzae* type b and their combinations (adsorbed vaccines), should not be frozen [1]. Exposure of vaccines to freezing temperatures is widespread and occurs across all segments of the cold chain [2–11]. The most recent systematic literature review highlights that in all segments of distribution between 75% and 100% of the vaccine shipments were exposed to freezing temperatures [12]. When a vaccine is damaged by freezing, physical properties as well as the immunological properties are adversely affected and the loss of potency can never be restored [13–16].

Use of vaccine affected by freezing can result in compromised immunogenicity in recipients and increases the local reactions such as sterile abscesses [17–19]. The lattice made up of bonds between the adsorbent and the antigen in aluminium-based freeze-sensitive vaccines is broken when the vaccine is affected by freezing [1]. Separated adsorbent tends to form larger, heavier granules that gradually settle at the bottom of the vial when this is shaken [1,20,21]. The size of the conglomerates seems to increase when freezing and thawing cycles are repeated [1]. Shake test, designed to determine whether adsorbed vaccines have been affected by freezing through observing the sedimentation rates in control (purposely frozen) and test vials, has been validated by the WHO in a recent study [20,21]. The validation study showed 100% positive predictive value and 100% specificity (95% CI: 0.99–1.00) and 100% sensitivity (95% confidence interval, CI: 0.97–1.00). In this study phase contrast microscopy was used as the golden test. In phase contrast microscopy, all vaccines affected by freezing showed conglomerates of large precipitates with crystalline structure while non-frozen vaccines showed fine-grain structure.

This experimental study was designed to further evaluate structural damages in adsorbed vaccines affected by freezing using scanning electron microscopy and X-ray analysis of the elements.

2. Methods

2.1. Sampling frame and temperature treatment of vaccines

All 42 samples tested in this experimental programme were selected randomly from 475 vials included in our previously developed experimental programme [20]. Eight types of WHO pre-

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qualified freeze-sensitive adsorbed vaccines (DTP, DT, dT, TT, DTP–HepB, HepB, DTP–HepB–Hib, Hib) from 10 manufacturers (Sanofi Pasteur, France; Serum Institute of India; CIGB, Cuba; GSK, Belgium; Berna Biotech, Republic of Korea; Merck, United States of America; Shanta Biotech, India; PT BioFarma, Indonesia; NCIPD, Bulgaria; Panacea Biotechnics, India) were investigated.

Original labels were removed and study labels containing a 7 digit number was applied to vials. Samples were grouped and treated at two different temperatures. For optimum storage, the first group of 21 vials used was kept continuously at +2 °C to +8 °C. The second group of 21 vials of the same type and manufacturer was exposed to −25 °C for a period of 24 h (solid frozen) at the Thermometry and Ionizing Radiation Section of the Federal Office of Metrology laboratories in Bern, Switzerland. Vaccines were then transported to Warsaw, Poland and were kept at +2 °C to +8 °C storage during the study.

2.2. Assay of the precipitation time of vaccines

Vials were vigorously shaken and the time of precipitation of all vaccines was measured until no further increase in the height of the sediment pillar was observed. The time was measured using an electronic timer. On the basis of the measurements, the precipitation coefficient was calculated (precipitation coefficient = full precipitation time for non-frozen vaccine/full precipitation time for frozen-damaged vaccine).

2.3. Scanning electron microscopy and X-ray analysis of the elements

For preparation of the samples to be examined under the scanning electron microscopy, 5 μl of each suspended vaccine was mounted onto the conductive table (specimen holder) covered with a carbon band. The samples were dried over 3 h at room temperature (20 °C). After drying, half of the samples were covered with a thin gold-layer using the Vacuum Evaporator JEE-4C, JEOL, Japan. The samples were observed using the scanning electron microscope Quanta 200, Czech Republic. All 42 samples were photographed at magnifications between 300x and 4000x. X-ray analysis of the sediment of vaccines (without gold-plating) was conducted using the Micro-analyser EDX.

3. Results

Scanning electron microscopy of vaccines kept at +2 °C to +8 °C showed uniform flocculent structure either dense (Fig. 1A) or dispersed (Fig. 1C). In lower magnification levels, no details of the

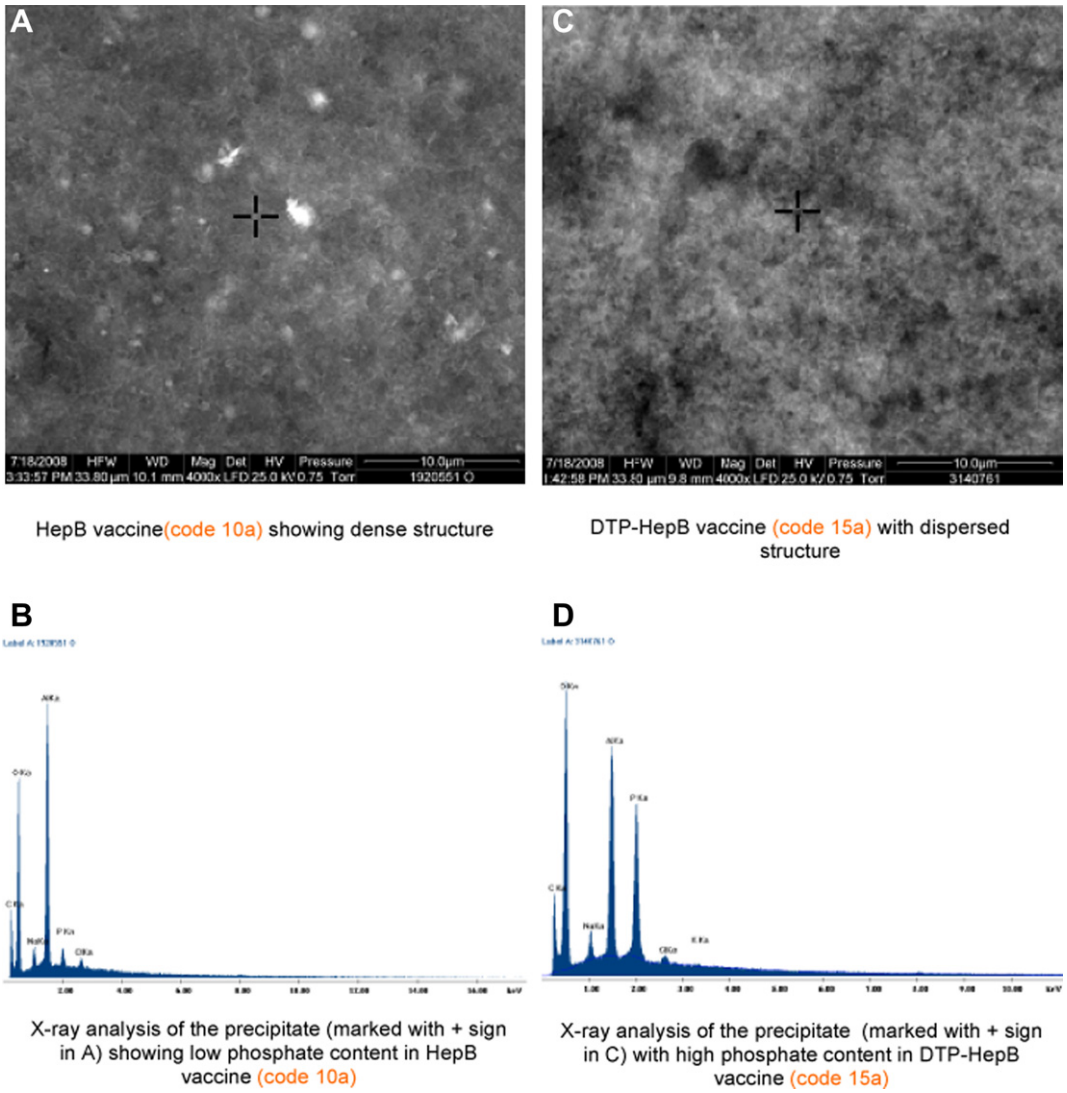

Fig. 1. Scanning electron micrographs and X-ray analysis of the elements of non-frozen HepB and DTP–HepB vaccines (kept at +2 °C to +8 °C at all times). HepB, hepatitis B; DTP, diphtheria–tetanus–pertussis.

structure was visible, this was the main reason we have increased the magnification levels to make structural formation visible. This was possible starting with 4000x magnification. X-ray analysis of the elements showed always high oxygen content along with aluminium and phosphate depending on the type of aluminium salt (aluminium hydroxide or aluminium phosphate).

Scanning electron microscopy of vaccines damaged by freezing (exposed to −25 °C for 24 h) exhibited conglomerates either with rough (Fig. 2A and C) or smooth surfaces (Fig. 2B and D).

X-ray analysis of precipitates in vaccines affected by freezing is displayed in Fig. 3. In all samples Aluminium was the highest content indicating the conglomerates are mainly aluminium clutters. Oxygen content in frozen samples were found to be lower compared to non-frozen samples of the same vaccines, mainly due to damaged structure of lattice.

Precipitation times of the samples are given in Table 1. Full precipitation time in vaccines kept at +2 °C to +8 °C varied between 30 and 130 min, while the values in vaccines affected by freezing were from 6 to 65 min. The value of the precipitation coefficient was from 2.0 to 15.0.

All values measured in X-ray analysis are given in Table 2. Based on the results of the scanning electron microscopy, X-ray analysis of the elements and the precipitation time, vaccines affected by freezing are divided into three groups. It should be noted that it is not possible to comment on the freezing status of the samples referring to weight percentages of elements in X-ray analysis. However, high aluminium content in frozen samples compared to non-frozen samples of same vaccines indicate that these sediments are simply made of aluminium that is separated from the lattice.

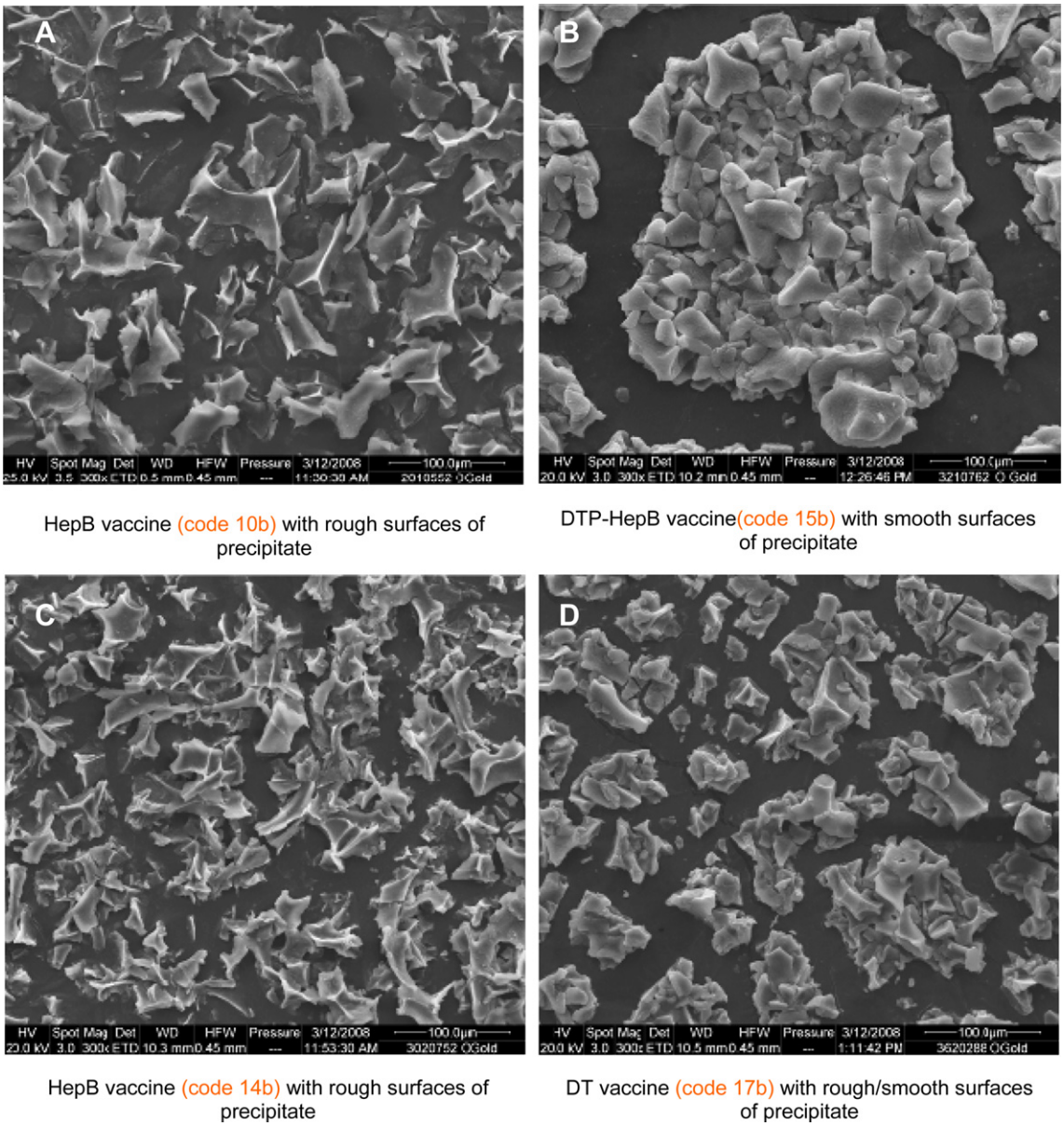

Fig. 2. Scanning electron micrographs of gold coated conglomerates of frozen HepB, DTP-HepB and DT vaccines (exposed to −25 °C for 24 h). HepB, hepatitis B; DTP, diphtheria–tetanus–pertussis; DT, diphtheria–tetanus.

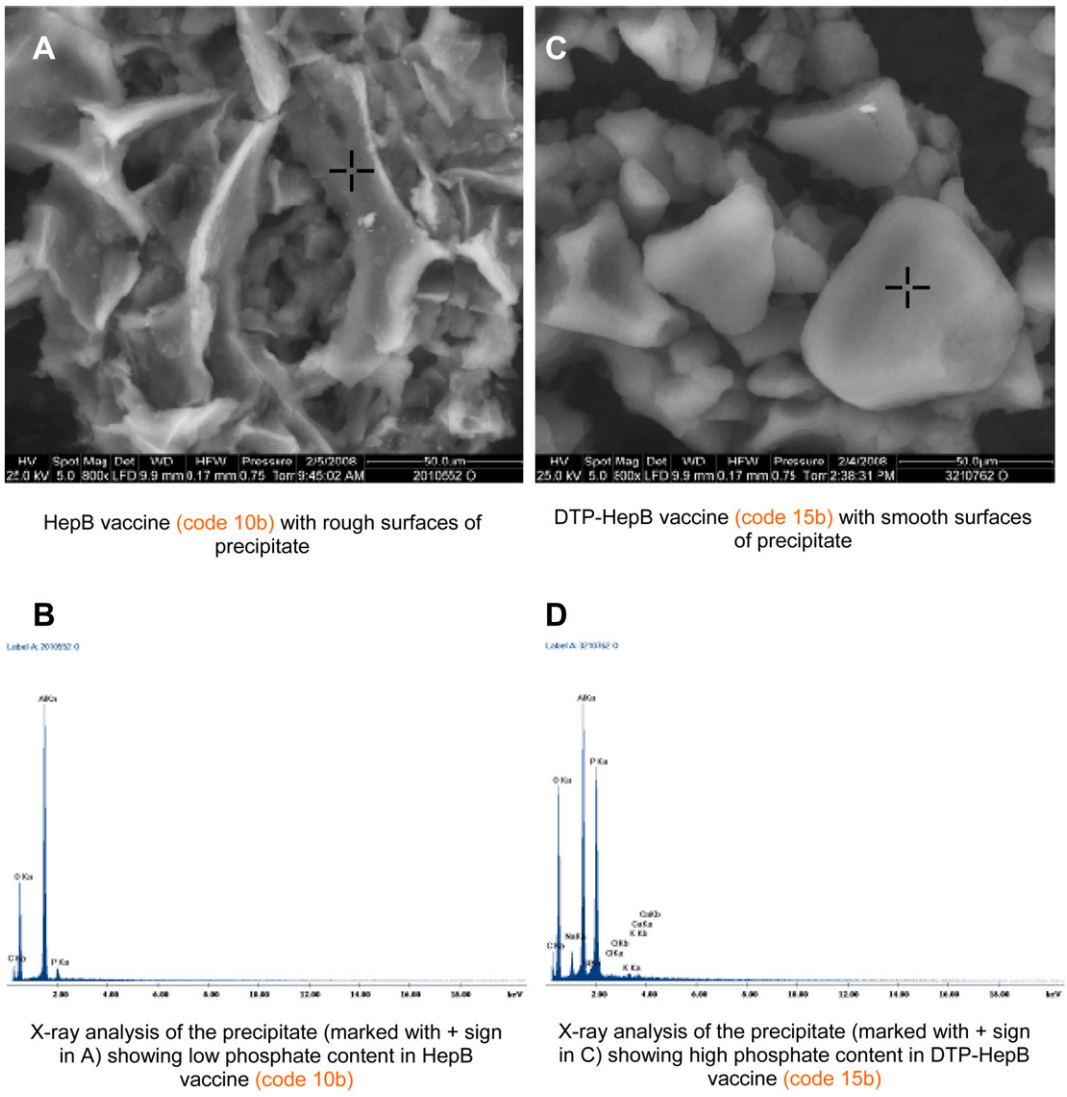

Fig. 3. X-ray analysis of the elements of frozen HepB and DTP-HepB vaccines (exposed to −25 °C for 24 h). HepB, hepatitis B; DTP, diphtheria–tetanus–pertussis.

4. Discussion

Randomly selected 21 non-frozen (kept at +2 °C to +8 °C) and 21 frozen (exposed to −25 °C for 24 h) adsorbed vaccines were examined under scanning electron microscopy and X-ray analysis was conducted for elemental analysis of precipitates.

Under the scanning electron microscopy, uniform flocculent structure was observed in vaccines kept at +2 °C to +8 °C, while vaccines affected by freezing showed broken structure with conglomerates of large precipitates. This was also confirmed in a recent study by Kartoglu et al., using phase contrast microscopy [20,21]. Conglomerates of large precipitates confirm that freezing breaks the lattice between the adsorbent and the antigen, leading the aluminium to form granules that grow in size. The uniform structure (either dense or dispersed) are not observed any more in adsorbed vaccines affected by freezing. Details of the structures in vaccines kept at +2 °C to +8 °C were only visible at higher magnification levels (4000x), but were never as sharp as in freeze-damaged samples. This was mainly due to having continuous mesh shape structure in vaccines kept at +2 °C to +8 °C. In freeze-damaged samples the whole lattice structure was broken and cluttered aluminium produced sharper images.

The shake test is based on the difference or similarity of sedimentation rates of purposely frozen a control vial and a test vial [1,20,21]. Adsorbed vaccines affected by freezing form heavy granular precipitates that sediment at a faster rate than lighter uniform flocculent structure of non-frozen vaccines. This difference was interpreted in Table 2 with precipitation coefficient. Frozen samples of same vaccines precipitated minimum of 2 times faster than their non-frozen equivalents. The highest precipitation coefficient was found in one of the DT vaccines (15 times faster). The precipitation time of vaccines affected by freezing is 4.5 times faster on average compared to non-frozen samples. Aleksandrowicz et al., also found similar results with aluminium hydroxide adjuvanted freeze-damaged vaccine samples 100% precipitating within 30 min while precipitation was at maximum of 57% level in 24 h in non-frozen samples. In this study the precipitation time was measured not in vials but in pipettes [22].

Based on the results of the scanning electron microscopy, X-ray analysis of the elements and the precipitation time of sediment, vaccines affected by freezing can be divided into three groups. The first group of vaccines can be characterized with a rough surface of conglomerates composed of precipitates with sharp edges (Figs. 2A, C and 3A); low in phosphate content (from

Table 1
Comparative analysis of precipitation time of non-frozen (kept at +2 °C to +8 °C at all times) and frozen vaccines (exposed to −25 °C for 24 h).

Type	Non-frozen vaccines		Freeze-damaged vaccines		Precipitation coefficient (non-frozen/damaged)
	Code	Precipitation time (min)	Code	Precipitation time (min)	
DTP	1a	120	1b	10	12
DT	2a	120	2b	8	15
dT	3a	120	3b	9	13.3
dT	4a	90	4b	25	3.6
TT	5a	90	5b	25	3.6
DTP-HepB	6a	100	6b	21	4.8
HepB	7a	30	7b	10	3
HepB	8a	30	8b	10	3
DTP-HepB	9a	30	9b	15	2
HepB	10a	60	10b	10	6
DTP-HepB −Hib	11a	45	11b	15	3
HepB	12a	90	12b	12	7.5
Hib	13a	35	13b	11	3.2
HepB	14a	60	14b	6	10
DTP-HepB	15a	130	15b	65	2
DTP	16a	130	16b	65	2
DT	17a	90	17b	20	4.5
TT	18a	70	18b	15	4.7
DT	19 a	90	19b	9	10
dT	20a	90	20b	7	12.9
TT	21a	80	21b	8	10

dT, diphtheria–tetanus (adult type); DT, diphtheria–tetanus (paediatric type); DTP, diphtheria–tetanus–pertussis; HepB, hepatitis B; Hib, *H. influenzae* type b; TT, tetanus toxoid.

0.72 Wt% to 5.00 Wt%; average 2.37 Wt%); short precipitation time (8.7 min − average of 10 vaccines) and high value of the precipitation coefficient (9.5 times faster − average of 10 analysis). The second group of vaccines can be characterized with a rough/smooth surface of massed precipitates (Fig. 2D); increased phosphate content (from 12.60 Wt% to 20.23 Wt%; average 17.72 Wt%); intermediate precipitation time (17.60 min − average of 7 vaccines) and a lower value of the precipitation coefficient (3.9 − average of 7 vaccines). The third group of freeze-damaged vaccines can be characterized with a smooth surface of conglomerates (Figs. 2B and 3C); high in phosphate content (from 20.62 Wt% to 24.23 Wt%; average 22.67 Wt%); long precipitation time (36.25 min − average of 4 vaccines) and the lowest value of the precipitation coefficient (2.1 − average of 4 vaccines). In all cases, X-ray analysis showed that these precipitates are mainly made of aluminium, confirming that what precipitates in vaccines affected by freezing is mainly cluttered aluminium. This was also confirmed by Aleksandrowicz et al., study [22].

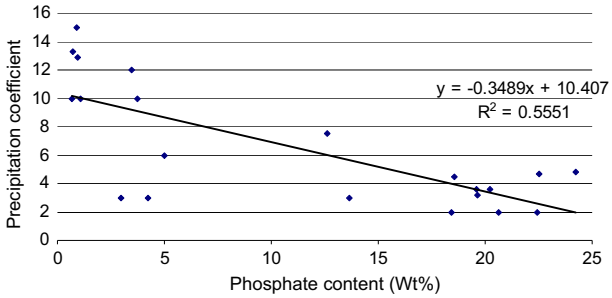

Fig. 4. Correlation between phosphate content (Wt%) in X-ray analysis and precipitation coefficient in adsorbed vaccines affected by freezing.

The correlation between Phosphate content in X-ray analysis (Wt%) and precipitation coefficient was analyzed in Fig. 4.

As seen in Fig. 4, low phosphate content in conglomerates is connected with the high values of the precipitation coefficient (R^2 = 0.56). The lower the phosphate content, the faster the precipitation rate. Phosphate content was also found to be related with formation of the precipitates, lower values are mostly resulted in rough surfaces with sharp edges while higher phosphate content affected precipitates' surfaces to be more smooth.

These findings, documenting the structural damages in adsorbed vaccines affected by freezing, confirm the value of the shake test in deciding whether aluminium-based freeze-sensitive vaccines have been affected by freezing.

Table 2
X-ray analysis of the elements in conglomerates in vaccines affected by freezing.

Group	Type of vaccines and code	X-ray analysis of the elements (Wt%)									
		O	Na	Mg	Al	Si	P	S	Cl	K	Ca
1	DTP (1b)	45.97	–	–	50.13	–	3.49	0.41	–	–	–
	DT (2b)	48.95	–	–	48.78	0.43	0.88	0.97	–	–	–
	dT (3b)	46.52	–	–	51.85	–	0.72	0.91	–	–	–
	HepB (7b)	48.10	0.51	–	47.15	–	4.24	–	–	–	–
	HepB (8b)	48.30	0.68	–	48.03	–	2.99	–	–	–	–
	HepB (10b)	40.98	–	–	54.03	–	5.00	–	–	–	–
	HepB (14b)	47.25	0.81	–	47.6	0.20	3.75	–	0.39	–	–
	DT (19b)	47.09	0.36	–	48.92	0.22	1.08	0.53	1.80	–	–
	dT (20b)	44.05	0.33	0.67	51.66	–	0.94	0.39	1.98	–	–
	TT (21b)	54.12	0.54	–	43.46	0.38	0.66	0.23	0.22	–	0.39
2	TT (5b)	54.56	2.88	–	22.95	–	19.61	–	–	–	–
	dT (4b)	53.05	4.05	–	22.67	–	20.23	–	–	–	–
	DTP-HepB (9b)	50.24	1.75	–	29.58	–	18.44	–	–	–	–
	DTP-HepB-Hib (11b)	52.97	–	–	33.38	–	13.65	–	–	–	–
	HepB (12b)	54.82	–	–	32.58	–	12.60	–	–	–	–
	Hib (13b)	53.77	3.55	–	22.49	–	19.64	–	0.20	0.34	–
	DT (17b)	54.84	3.47	–	22.58	0.38	18.58	–	0.16	–	–
3	DTP-HepB (6b)	47.47	2.35	–	25.94	–	24.23	–	–	–	–
	DTP-HepB (15b)	48.76	3.56	–	23.85	0.40	22.41	–	0.25	0.48	0.31
	DTP (16b)	52.20	2.72	–	23.66	0.65	20.62	–	0.16	–	–
	TT (18b)	49.98	2.15	–	24.19	0.51	22.54	–	–	0.13	0.50

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Competing interests

None declared.

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