



Haemophilus influenzae Type b Immunity After Hib Vaccination and Its Association with Serum Iron, Zinc, and Copper in Southwest Iran: Is a Vaccine Booster Needed?

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Abstract

Background: Globally, vaccination has substantially reduced invasive *Haemophilus influenzae* type b (Hib) disease. However, limited information is available on the persistence of protective antibodies after Iran incorporated the Hib-containing pentavalent vaccine into the national immunization program in 2014. Micronutrients, including iron, zinc, and copper, may influence immune responses.

Objectives: This study aimed to assess Hib-specific antibody levels and their association with trace element status in vaccine-eligible children in Jahrom, Iran.

Methods: This cross-sectional study was conducted in Jahrom, southwestern Iran, from October 2024 to May 2025. Serum samples were collected from 450 children who had received the Hib-containing pentavalent vaccine after its introduction in Iran in 2014, with doses administered at 2, 4, and 6 months of age. Anti-Hib immunoglobulin G (IgG) antibody concentrations were measured using an enzyme-linked immunosorbent assay (ELISA).

Results: Among 450 children, 246 (54.7%) were male, and the mean age was 78.4 ± 33.2 months. Overall, 75.6% had short-term Hib protection, and 18.7% had long-term protection. The mean serum anti-Hib antibody concentration was 0.547 ± 0.446 $\mu\text{g/mL}$. Linear regression (adjusted $R^2 = 0.883$) showed that serum concentrations of iron ($B = 0.009$, $P < 0.001$), zinc ($B = 0.001$, $P = 0.010$), and copper ($B = 0.012$, $P < 0.001$) were positively associated with anti-Hib antibody levels.

Conclusions: Following the introduction of the Hib-containing pentavalent vaccine, only 18.7% of participants had antibody titers indicating sustained protection. Micronutrients may influence vaccine-induced immunity. These findings underscore the need for rigorous surveillance and reporting of invasive Hib disease to assess the disease burden despite vaccination. The low proportion of children with long-term protective antibody levels suggests that supplemental Hib vaccination may be needed to ensure durable protection.

Keywords: Hib Antibody, Vaccine, Iron Deficiency, Mineral

1. Background

Haemophilus influenzae type b (Hib) strains are among the most important bacterial causes of diseases

such as epiglottitis. Almost exclusively in children younger than 5 years, Hib causes pneumonia and meningitis (1, 2). Before the widespread implementation of vaccination in 2000, Hib accounted for an estimated

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8.13 million severe infections in children younger than 5 years and caused approximately 371,000 deaths globally (3).

A primary approach to reducing the risk of infections, particularly Hib infection, is immunization through vaccination (4). Vaccination has substantially reduced invasive Hib disease worldwide (3, 5, 6). The prevalence of invasive Hib infections in infants and young children has decreased by approximately 99% since the introduction of Hib conjugate vaccines in 1988 to fewer than 1 case per 100,000 children younger than 5 years (7). In Iran, Hib caused approximately 12.8% and 3.5% of meningitis cases in children younger than 5 years before and after Hib vaccine introduction, respectively (8, 5). In 2014, Iran introduced the DTPw-HepB-Hib pentavalent vaccine, consisting of whole-cell diphtheria-tetanus-pertussis, hepatitis B, and Hib, with the polyribosylribitol phosphate component conjugated to tetanus toxoid, into its routine vaccination program, replacing the former DTPw and hepatitis B vaccines. The recommended doses are administered at 2, 4, and 6 months of age (5). In 2008, before Hib vaccine use in Iran, nearly 85% of children younger than 5 years living in Jahrom had immunity against Hib, and 69.2% had long-term protection, indicating exposure to Hib bacteria in their living environment (9).

Minerals, particularly zinc, are among the factors affecting optimal immune system function and vaccine responses (10). Zinc (Zn), copper (Cu), and iron (Fe) deficiencies weaken the immune system (11, 12) because of their substantial effects on B- and T-cell proliferation (13-16). Conversely, individuals with iron overload conditions are also at higher risk of developing infections (17).

2. Objectives

Despite global evidence showing that Hib vaccines markedly reduce invasive infections, information on the persistence of protective antibodies after Iran adopted the pentavalent vaccine remains insufficient. Although pre-vaccination studies in Jahrom indicated relatively high natural exposure and immunity to Hib, no comprehensive evaluation has assessed the magnitude and durability of vaccine-induced immunity in Iranian children. Furthermore, the potential influence of trace elements, including zinc, copper, and iron, which play critical roles in immune regulation and vaccine responsiveness, has not been systematically investigated in this context. Addressing this gap is essential to assess the effectiveness of the current vaccination strategy, identify potentially modifiable factors influencing

immune protection, and provide evidence to inform policy decisions regarding the need for booster doses in the Iranian immunization program. Therefore, this study was conducted to determine the level of Hib-specific antibody protection among children in Jahrom, Iran, who were eligible for vaccination after the introduction of the pentavalent vaccine, and to investigate the possible association between antibody responses and trace elements, including zinc, copper, and iron.

3. Methods

3.1. Study Design and Setting

This cross-sectional study was conducted in Jahrom, southwest Iran, from October 2024 to May 2025. A total of 450 children who had received the complete pentavalent DTPw-HepB-Hib vaccination schedule at 2, 4, and 6 months of age were included.

The sample size was calculated using a standard formula, assuming a prevalence of 50%, a 95% confidence level, and a 5% margin of error. To account for potential missing data, the sample size was increased to 450 participants.

3.2. Participants

The inclusion criterion was children born after September 19, 2014, who had been fully vaccinated against Hib.

The exclusion criteria were as follows: 1) receipt of immunosuppressive drugs during the previous month; 2) diabetes mellitus, beta thalassemia major, or other chronic diseases; and 3) dialysis. All enrolled children had documentation indicating that they had received all three doses of the pentavalent vaccine.

3.3. Variables and Data Collection

The primary outcome was the serum concentration of anti-Hib IgG antibodies. Independent variables included age, sex, and serum concentrations of iron, zinc, and copper. Demographic and vaccination information was obtained from medical records.

3.4. Measurement of Outcomes and Exposures

Serum samples were tested for anti-polyribosylribitol phosphate antibodies at the Jahrom University of Medical Sciences Research Center using the IBL ELISA kit (Germany; Ref: RE56351), according to the manufacturer's instructions. Antibody levels < 0.15 µg/mL were classified as nonprotective, levels from 0.15

to $< 1 \mu\text{g/mL}$ as short-term immunity, and levels $\geq 1 \mu\text{g/mL}$ as long-term protection. Serum iron, copper, and zinc levels were measured using a spectrophotometric method. All serum samples were collected after 8 - 10 hours of fasting.

3.5. Statistical Analysis

The chi-square test was used to examine differences in proportions according to age, sex, and blood concentrations of iron, zinc, copper, and anti-Hib antibody. The independent t-test and one-way analysis of variance were used to analyze differences in mean values. Linear regression analysis was used to evaluate the association between anti-Hib antibody levels and age, sex, and serum levels of iron, zinc, and copper. All analyses were performed using SPSS version 16 (SPSS Inc., Chicago, IL, USA), and P values < 0.05 were considered statistically significant.

4. Results

Four hundred fifty children participated in the study, of whom 246 (54.7%) were male (Table 1). The mean age was 78.39 ± 33.15 months (range, 20 - 126 months). In addition, 318 participants (70.6%) were older than 60 months. The mean serum concentrations of iron, zinc, and copper were $62.46 \pm 30.98 \mu\text{g/dL}$ (range, 16 - 145), $101.99 \pm 37.45 \mu\text{g/dL}$ (range, 19 - 162), and $94.40 \pm 15.36 \mu\text{g/dL}$ (range, 75 - 130), respectively.

Table 1. Characteristics of Study Participants

Variables and Categories	Values ^a
Sex	
Male	246 (54.7)
Female	204 (45.3)
Age group (mo)	
20 - 36	71 (15.8)
37 - 60	61 (13.5)
61 - 84	121 (26.9)
85 - 108	64 (14.2)
109 - 126	133 (29.6)
Serum iron ($\mu\text{g/dL}$)	62.46 ± 30.98
Serum zinc ($\mu\text{g/dL}$)	101.99 ± 37.45
Serum copper ($\mu\text{g/dL}$)	94.40 ± 15.36
Serum Hib antibody ($\mu\text{g/mL}$)	0.547 ± 0.446

Abbreviation: Hib: *Haemophilus influenzae* type b.

^a Values are expressed as No. (%) or mean $\pm$ SD.

The mean serum Hib antibody concentration was $0.547 \pm 0.446 \mu\text{g/mL}$ (range, 0.06 - 1.58), with no

statistically significant difference between males and females (0.579 ± 0.472 and $0.510 \pm 0.411 \mu\text{g/mL}$, respectively; $P = 0.098$) (Table 2). The mean serum Hib antibody concentration was associated with the time since the last Hib vaccine dose administered at 6 months of age ($P = 0.009$). The lowest mean value was observed 97 - 108 months after the last vaccine dose (0.342 ± 0.265), and the highest mean value was observed 25 - 36 months after the last vaccine dose (0.676 ± 0.475). The mean serum Hib antibody concentration 97 - 108 months after the last vaccine dose was significantly lower than that at 14 - 24 months ($P = 0.049$), 25 - 36 months ($P < 0.001$), 73 - 84 months ($P = 0.004$), and 109 - 120 months ($P = 0.001$) after the last vaccine dose. In addition, the mean serum Hib antibody concentrations at 37 - 48 months ($P = 0.034$) and 49 - 60 months ($P = 0.015$) after the last vaccine dose were significantly lower than that at 25 - 36 months after the last vaccine dose.

Most participants (75.6%) showed evidence of short-term immunity against Hib. Only a small proportion (18.7%) had sustained immunity against Hib. Although boys had long-term immunity to Hib more often than girls, the difference was not statistically significant (22.4% vs. 14.2%; $P = 0.051$) (Table 2). The level of immunity to Hib was significantly associated with the time since receipt of the third Hib vaccine dose ($P = 0.006$).

Overall, 25.3% and 5.3% of children had low and high serum iron levels, respectively. In addition, 18.4% and 35.8% of participants had low and high serum zinc levels, respectively. No participant had high serum copper levels, and only 15.1% had low serum copper levels.

No child with low serum iron was classified as having long-term immunity, and no child with a serum iron level of $40 \mu\text{g/mL}$ or higher had Hib antibody levels lower than $0.15 \mu\text{g/mL}$ (Table 3). In contrast, 95.8% of children with high serum iron levels were classified as having long-term immunity ($P < 0.001$). Participants with Hib antibody levels $\geq 1.0 \mu\text{g/mL}$ had higher mean serum concentrations of iron, zinc, and copper than those in the other groups ($P < 0.001$ for all) (Table 3).

In the linear regression analysis, serum anti-Hib antibody levels were significantly associated with serum concentrations of iron ($P < 0.001$), zinc ($P = 0.010$), and copper ($P < 0.001$) (Table 4). In other words, with each unit increase in serum iron, zinc, and copper levels, Hib antibody levels also increased, although these increases were small, especially for zinc. The model fit was good (adjusted R-squared = 0.883).

5. Discussion

Table 2. Serum Anti-Hib Antibody Concentrations in Children According to Time Since Receiving Third Dose of Hib Vaccine and Sex

Variables and Category	No. (%)	Mean Concentration of Anti-Hib Antibody, µg/mL (SD)	< 0.15	0.15 - < 1.00	≥ 1.00
Time since last Hib vaccine dose at age 6 months (mo)					
14 - 24	39 (8.7)	0.536 (0.444)	5 (5.2)	71 (73.2)	21 (21.6)
25 - 36	58 (12.9)	0.676 (0.475)			
37 - 48	29 (6.4)	0.463 (0.381)	3 (4.2)	60 (83.3)	9 (12.5)
49 - 60	43 (9.6)	0.460 (0.366)			
61 - 72	44 (9.8)	0.507 (0.457)	13 (12.2)	71 (67.0)	22 (20.8)
73 - 84	62 (13.8)	0.596 (0.483)			
85 - 96	27 (6.0)	0.510 (0.426)	3 (4.4)	59 (86.8)	6 (8.8)
97 - 108	41 (9.1)	0.342 (0.265)			
109 - 120	107 (23.7)	0.616 (0.481)	2 (1.9)	79 (73.8)	26 (24.3)
P-value	0.009	0.009	0.006	0.006	0.006
Sex					
Male	246 (54.7)	0.579 (0.472)	11 (4.4)	180 (73.2)	55 (22.4)
Female	204 (45.3)	0.510 (0.411)	15 (7.4)	160 (78.4)	29 (14.2)
P-value	0.098	0.098	0.051	0.051	0.051

Abbreviation: Hib: *Haemophilus influenzae* type b.**Table 3.** Serum Anti-Hib Antibody Concentrations in Children Receiving Three Doses of Hib Vaccine According to Serum Iron, Zinc, and Copper Levels

Variables and Category	Mean Concentration of Anti-Hib Antibody, µg/mL (SD)	< 0.15	0.15 - < 1.00	≥ 1.00	P-Value
Serum iron level (µg/dL)					
< 40	0.187 (0.061)	26 (22.8)	88 (77.2)	0 (0.0)	< 0.001
40 - 120	0.609 (0.411)	0 (0.0)	251 (80.4)	61 (19.6)	
> 120	1.455 (0.151)	0 (0.0)	1 (4.2)	23 (95.8)	
P-value	< 0.001	-	-	-	-
Serum zinc level (µg/dL)					
< 70	0.203 (0.189)	26 (31.3)	55 (66.3)	2 (2.4)	< 0.001
70 - 120	0.549 (0.409)	0 (0.0)	171 (83.0)	35 (17.0)	
> 120	0.722 (0.483)	0 (0.0)	114 (70.8)	47 (29.2)	
P-value	< 0.001	-	-	-	-
Serum copper level (µg/dL)					
< 80	0.204 (0.104)	20 (29.4)	48 (70.6)	0 (0.0)	< 0.001
80 - 160	0.609 (0.456)	6 (1.6)	292 (76.4)	84 (22.0)	
P-value	< 0.001	-	-	-	-
Iron (µg/dL)		24.77 (5.40)	53.68 (21.17)	109.63 (18.39)	< 0.001
Zinc (µg/dL)		30.08 (4.92)	102.41 (34.18)	122.55 (27.56)	< 0.001
Copper (µg/dL)		78.27 (3.74)	89.63 (9.62)	118.73 (10.97)	< 0.001

Abbreviation: Hib: *Haemophilus influenzae* type b.

Based on these results, despite routine vaccination, only 18.7% of participants achieved antibody levels corresponding to long-term protection. This finding indicates that a substantial proportion of children may have suboptimal immunity several years after vaccination. Furthermore, the positive associations observed between anti-Hib antibody levels and serum

concentrations of iron, zinc, and copper suggest that micronutrient status may play a role in sustaining vaccine-induced immunity. Collectively, these results provide important insights into the current status of Hib protection in the study population and highlight potential considerations for public health strategies aimed at maintaining long-term immunity.

Table 4. Association of Hib Antibody Levels with Serum Concentrations of Iron, Zinc, and Copper Using Linear Regression ^a

Variables	Unstandardized Coefficient (B)	Lower Bound	Upper Bound	P-Value
Constant	-1.044	-1.149	-0.901	
Iron (µg/dL)	0.009	0.008	0.010	< 0.001
Zinc (µg/dL)	0.001	-0.001	0.001	0.010
Copper (µg/dL)	0.012	0.010	0.013	< 0.001
Time since last dose of vaccine	0.001	< 0.001	< 0.001	0.209

^aAdjusted R-squared = 0.883

Hib immunization has been shown to provide effective protection in children without underlying health conditions (18). The collective results of clinical trials (19) and extensive vaccination programs across multiple populations (20, 21) suggest that more than 90% of vaccinated individuals develop protective immunity. In England, the annual rate of Hib disease was 30 and 0.7 per 100,000 unvaccinated and vaccinated children aged 5 - 71 months, respectively (22).

Consistent with previous research, our findings confirm the immunogenicity of the Hib vaccine in children. Among participants for whom up to 10 years had passed since Hib vaccination, 94.2% had anti-Hib antibody levels of 0.15 µg/mL or higher. According to Matos et al., 97.25% of infants receiving the DTP-Hib vaccine generated high concentrations of polyribosylribitol phosphate IgG antibodies (≥ 1.0 µg/mL), which are considered indicative of long-term protection (23). Evidence from England indicates that Hib antibody concentrations measured before vaccination at two months of age averaged 0.37 µg/mL. After immunization, mean levels rose to 0.88 µg/mL at 12 months and 1.06 µg/mL at 43 months but declined to 0.51 µg/mL by 72 months, corresponding to an approximate 98% decrease in antibody levels across the follow-up period (22). Corad et al. showed that 93% of vaccinated children aged 24 months had protective levels of anti-Hib antibody (≥ 1.0 µg/mL) (24). As reported in other research, primary immunization significantly affected anti-Hib antibody concentrations (22). In our study, the change in mean anti-Hib antibody concentrations and the trend in the proportion of children with anti-Hib concentrations below 0.15 µg/mL, a putative protective threshold, were statistically significant. In England, 51% and 43% of children aged 12 months were classified as having short-term and long-term protection, respectively (22). In addition, the frequencies of short- and long-term protection at 43 months were 41% and 51%, respectively, and those at 72 months were 39% and 21%, respectively (22).

After three vaccine doses, approximately 40% of infants reach antibody levels of 1.0 µg/mL, whereas 70% reach 0.15 µg/mL (19). In contrast to these findings, our study showed that 21.6% of children aged 20 - 42 months (14 - 36 months after the last vaccine dose) had Hib antibody concentrations ≥ 1.0 µg/mL, and 95.8% had concentrations ≥ 0.15 µg/mL.

After Hib vaccination, 94.2% of children had at least short-term protection, but 81.3% had antibody concentrations below the threshold for long-term immunity.

A 15-year Hib surveillance study in Kenya showed that, eight years after vaccination, 79% of children aged 4 - 35 months in the risk group had antibodies reflecting long-lasting immunity (25). However, our study showed that only 21.6% of children aged 20 - 42 months had sufficient Hib antibody levels. In The Gambia, the primary three-dose Hib vaccine remained highly effective in controlling invasive disease even 13 years after its introduction (26). Decreasing hepatitis B surface antibody levels with age in a study by Bakhshipour et al. indicated that routine childhood vaccination programs are inadequate for preventing hepatitis B virus transmission and that changes in vaccine routes or additional booster vaccination may be essential (27).

Serum anti-Hib antibody concentrations were associated with serum levels of iron, zinc, and copper. Accordingly, children with higher serum levels of iron, zinc, and copper had higher serum anti-Hib antibody concentrations. In addition, nearly 23.0% of children with low serum iron had anti-Hib antibody levels lower than 0.15 µg/mL, indicating insufficient immunity. No child with a serum level higher than 40 mg/dL was classified as having insufficient immunity. In a study of individuals aged 65 years or older without coronavirus disease 2019, antibody concentrations after two vaccine doses were significantly lower in vaccine recipients with iron deficiency than in those without iron deficiency (28). One study showed that patients with iron-deficiency anemia had lower anti-Hib IgG

concentrations in their blood (29). In a Chinese population aged ≥ 10 years, iron-deficient individuals showed decreased levels of measles-specific IgG antibodies compared with peers with normal iron status (15). Evidence from Kenya showed that iron deficiency during vaccination predicted diminished responses to diphtheria, pertussis, and pneumococcal vaccines, whereas iron supplementation at the time of measles immunization may enhance the primary antibody response (30). In contrast, another study observed no strong association between iron deficiency and the effectiveness of diphtheria, tetanus (31), and typhoid (32) vaccines. Furthermore, a large retrospective cohort study found that COVID-19 vaccine effectiveness over the two-dose period was nearly the same in participants with and without iron deficiency (91.9% vs. 92.1%) (33).

Lower serum zinc levels were associated with higher tetanus vaccine titers, but no association was found with measles, rotavirus, pertussis, or polio vaccine responses (34). In adults older than 65 years, anti-influenza antibody levels were independent of serum zinc concentrations (35).

Zinc deficiency may reduce antibody production by impairing B-cell maturation (13). Similar effects are observed with iron and copper deficiencies, which impair B-cell proliferation (16) and B-cell function (14), respectively. Iron deficiency has been shown to reduce B-cell growth, T-cell function, and adaptive immune responses (36).

In children who had previously received Hib vaccination, booster doses increased antibody levels and seroprotection (37). In another study, an additional Hib vaccine dose was administered to asplenic individuals whose antibody concentrations after vaccination were lower than 1.0 $\mu\text{g/mL}$. In that study, revaccination increased antibody titers that provided protection (38).

5.1. Strengths and Limitations

This study provides novel, region-specific data on the long-term persistence of Hib-specific antibodies years after routine vaccination in Iran, contributing to an understanding of the long-term effectiveness of the national immunization program. The simultaneous measurement of micronutrient status, including iron, zinc, and copper, and its correlation with antibody titers is another strength, as it helps characterize determinants of vaccine-induced immunity.

However, this study has some limitations. First, its cross-sectional design precludes causal interpretation of the associations between micronutrient status and long-term antibody persistence; therefore, longitudinal

or interventional studies are recommended to explore causality. Second, the study did not account for nutritional status, such as height/weight and anemia, breastfeeding history, socioeconomic status, or recent infections, which may influence both micronutrient levels and immune responses. Third, the study was conducted in a single region, and the generalizability of the results may be limited in other regions of Iran or other populations. Fourth, other predictors of immune responses, such as genetic determinants, underlying illness, or exposure history to Hib, were not accounted for. Fifth, vaccination history was recorded based on the memory of children's mothers, which may be inaccurate.

5.2. Conclusions

After the introduction of the Hib-containing pentavalent vaccine, only 18.7% of children achieved long-term protective antibody levels. Serum iron, zinc, and copper concentrations were positively associated with anti-Hib antibody levels, suggesting that trace element status may influence vaccine-induced immunity. These results underscore the need for ongoing monitoring of Hib immunity and consideration of nutritional factors when evaluating long-term protection and potential booster vaccination strategies.

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Footnotes

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responsible for data collection and sample acquisition. E.S.J. also carried out the laboratory analyses. N.R. data collection and sample acquisition. All authors read and approved the final version of the manuscript.

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