Biologicals 40 (2012) 262–265

Contents lists available at SciVerse ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Significance of circulating toxin and antitoxin in unimmunized tetanus cases of neonates and infants

S.S. Grover*, S.S. Negi [1], S. Singh [2], K. Ray [3]

National Centre for Disease Control, (NCDC), 22-Sham Nath Marg, Delhi 54, India

ARTICLE INFO

Article history:
Received 25 October 2011
Received in revised form
1 March 2012
Accepted 3 March 2012

Keywords:
Tetanus toxin
Antitoxin
Neonates
Infant

ABSTRACT

The level of circulating tetanus toxin, antitoxin and their individual influence on the outcome of tetanus cases were determined in unimmunized 125 neonatal and 39 infant cases of tetanus. PHA (passive haemagglutination) test showed 40% positive cases for toxin while its absence in the remaining cases indicated of either toxin fixation to the central nervous system (CNS) or it got neutralized by antitoxin. TN (toxin neutralization) and PHA test carried out in 46 sera samples revealed a strong positive correlation ($r = 0.9$) showing that 35/46 (76%) and 38/46 (82.6%) samples were positive for antitoxin, respectively. 25.4% of the neonate and infant cases and 34% of the control group had a protective serum tetanus antitoxin level. 42.5% of the paired sera from unimmunized mothers and their neonates showing nonprotective antitoxin levels suggested that a high level of antitoxin is needed for transplacental transfer, although transfer may not play a decisive role in the resistance against the disease. The presence of toxin or antitoxin in the clinical cases did not affect the outcome of the disease, although in neonates, presence of toxin was found to be a bad prognostic sign. This study explicitly advocates for the need to improve the vaccination coverage strategy.

1. Introduction

Tetanus, though vaccine preventable, still remains a fatal childhood disease of global importance. It is caused by the release of the tetanospasmin neurotoxin of *Clostridium tetani*, causing high mortality varying from 40 to 80% [1]. In neonatal tetanus cases, even with treatment, the case fatality rate can be 80–90%, accounting for significant preventable deaths [1]. WHO estimated that 59,000 newborns died from neonatal tetanus in 2009 [2]. Among the developing world, 73% of neonatal tetanus deaths occur in eight countries, of which India has the highest percentage [3]. Adequate immunity acquired from a full course of diphtheria and tetanus immunization wanes in late childhood and adolescence. Periodic boosters of diphtheria and tetanus toxoids, first at 18 months (DPT), then between 54 and 72 months (DT), followed by two doses of tetanus toxoid (TT) at the ages of 10 and 16 years, is warranted for persistence of adequate immunity [4]. Measuring tetanus antitoxin in human sera is frequently required in antibody surveys for national immunization strategy and in investigation of immunodeficiency. Moreover, measuring tetanus antitoxin is of paramount importance in assessing the immune status of individuals at risk of tetanus infection while monitoring the efficiency of mass vaccination programs. In the present communication, we measured the level of circulating toxin and antitoxin in neonatal and infant tetanus cases from Delhi and the influence of these parameters on the clinical outcome of these cases, in order to understand the persistence of a paediatric tetanus even after years of introduction of a national mass immunization program.

2. Materials and methods

Blood samples from one hundred and sixty four tetanus cases were received from different hospitals in Delhi during 2006–2007. Of the 164 cases, 125 were neonates and 39 were infants. All the necessary clinical details and patient consent were obtained from the referring hospital in the prescribed format provided by us. Convalescent blood samples were available after two weeks from 15 cases only. In 54 neonatal cases, maternal blood was also collected. None of the tetanus cases were treated with tetanus antitoxin at the time of blood collection. Neither the patients nor their mothers had any history of active or passive tetanus

* Corresponding author. Division of Biochemistry & Biotechnology, National Centre for Disease Control (NCDC), 22-Sham Nath Marg, Delhi 110054, India. Tel.: +91 9313101019; fax: +91 11 23912833.
E-mail addresses: grovernicd@yahoo.co.in (S.S. Grover), negidr@yahoo.co.in (S.S. Negi), satyenders@gmail.com (S. Singh), drkray@yahoo.com (K. Ray).
[1] Present address: National Institute of Biologicals, Noida, Sector-62, India.
[2] Present address: College of Pharmacy, Pt B D Sharma University of Health Sciences, Rohtak, Haryana, India.
[3] Present address: Department of Microbiology, MRDC, Faridabad, Haryana, India.

1045-1056/$36.00
doi:10.1016/j.biologicals.2012.03.001

immunization. After separation, the serum samples were stored at −20 °C until further use. Tetanus toxin level was estimated in all 164 sera. Antitoxin level, however, was determined only in 110 serum samples (73 neonates and 37 infants) due to insufficient sample quantity in the remaining cases. Convalescent sera of 15 cases were studied both for toxin and antitoxin level.

Controls included 288 normal infants and children (2 months–5 years) without any history of tetanus immunization. Their sera samples were screened for the presence of antitoxin. Fifty of these sera were also tested for the presence of toxin.

2.1. Estimation of tetanus antitoxin

The level of tetanus antitoxin was determined by PHA, as previously described by Hardegree et al. [5], with certain modifications, as previously published by us [6]. Among 110 test and 288 normal serum samples, 46 samples selected randomly were also tested by toxin neutralization test to determine the correlation and efficacy of the two test using the method described earlier by Barile et al. [7]. Briefly, TN test was performed in reaction volume of 50 μl containing fixed amounts of purified tetanus toxin and serial two-fold dilutions of patient sera (antitoxin) in diluent buffer [1 mg/ml bovine serum albumin (Sigma chemicals), 0.5% vol/vol heat inactivated normal rabbit serum (Life Technologies) and 0.01% wt/vol Merthiolate (Sigma chemicals)], then incubated for 2 h at room temperature before the addition of 50 μl of a 0.2% suspensions of antitoxin sensitized sheep erythrocytes in the same diluent. After mixing the samples and sealing the plates, they were incubated at 4 °C overnight before observing the agglutination patterns. The standard tetanus toxin and antitoxin were received from Central Research Institute, Kasuali, Himachal Pradesh, India in lyophilized form. The L+/1000 dose of this toxin determined in our laboratory was 4.5 μg. The sera were diluted in peptone water by two-fold dilutions. In both tests, sera showing ≥0.015 unit/ml of antitoxin were considered to have protective immunity (positive) and those below that level were considered negative as previously shown [5–8].

2.2. Estimation of tetanus toxin

Toxin level was determined by PHA test as described by Holmes and Parlow [9]. Briefly, formalinized tanned sheep erythrocytes were coated with standard tetanus antitoxin by mixing four volumes of phosphate buffered saline (PBS pH 6.4), 1 volume of 10% formalinized tanned sheep erythrocytes and 0.5 volume of tetanus antitoxin (10 I.U./ml). The mixture was incubated at room temperature for 1 h with constant shaking on a rotator. The suspension was centrifuged for 15 min at 270 × g at 4 °C. The supernatant was discarded and the cells were washed three times with 50 ml normal saline. Finally, the packed cells were resuspended at a final concentration of 2% (vol/vol) in phosphate buffered saline pH 7.4 containing 0.1% bovine serum albumin and 0.01% thiomersal. The antitoxin sensitized cells were distributed in 1 ml aliquots and stored at −70 °C.

The PHA test was carried out in disposable sterile plastic microtiter plates with V bottom wells (Nalge Nunc International Co., Naperville, USA). Phosphate buffered saline (pH 7.4) containing 0.5% vol/vol heat inactivated normal rabbit serum was used as the diluent. The test sera were diluted two-fold and titration was carried out in a 50 μl volume containing 0.1% (vol/vol) antitoxin sensitized sheep erythrocytes. Negative controls for all the experiments included antitoxin sensitized cells incubated in the absence of toxin and un-sensitized control cells incubated in the presence of serial two-fold dilutions of standard tetanus toxin. A positive control was also set up with serial two-fold dilutions of standard tetanus toxin (protein content of 15.6 mg/ml) after a proper initial dilution in the presence of antitoxin sensitized cells.

After preparing the reaction mixtures, the plates were sealed and incubated at +4 °C overnight before observing the agglutination pattern. The result was expressed in nanograms (ng) of toxin protein by comparison with the standard toxin. Sera showing no haemagglutination at 1:2 dilution were considered negative for toxin.

2.3. Statistical analysis

Chi square test with Yates modification was used to compare the positivity rates of PHA and TN test.

3. Results

The results of tetanus antitoxin estimation by TN and PHA (Table 1) in 46 sera samples showed a high correlation co-efficient ($r = 0.8965$), indicating strong concordance between the two tests. Further analysis showed that the two tests had similar titres in 47.8% of cases. PHA gave higher and lower titres than TN in 26% of cases each.

The tetanus antitoxin levels of control group and tetanus cases did not appear to be different except that a slightly higher proportion of the control group had a protective antitoxin level. In neonatal and infant tetanus cases, this level was seen in 23.3 and 29.7% of cases respectively with no significant statistical difference ($\chi^2 = 0.53$, $p > 0.05$)(Table 2).

Table 1
Comparison of toxin neutralization (TN) and passive haemagglutination test (PHA) for estimating tetanus antitoxin.

	Passive haemagglutination (I.U.)											Total	
	Antitoxin range		<0.015	0.015–<0.1			>0.1–<1.0				>1.0		
				0.015	0.03	0.06	0.125	0.25	0.5	1.0	−2.0		
Toxin neutralization(I.U.)	<0.015		7	4								11	11
	0.015–<0.1	0.015	–	6								6	
		0.03	1	–	2	2						5	18
		0.06		2	2	2		1				7	
	>0.1–<1.0	0.125			1			1	1			3	
		0.25						2				2	
		0.5					1	3	1	3		8	14
		1.0								1		1	
	>1.0–2.0							1		1	1	3	3
	Total		8	12	5	4	1	8	2	5	1	46	46
			8		21			16			1		

Table 2
Tetanus antitoxin titre in normal and tetanus cases by PHA test.

Group	No. tested	Antitoxin titre I.U./ml.				Total of titre > 0.015
		Number (%) <0.015	0.015–0.125	>0.125–1.0	>1.0	Number (%)
Normal infants and children	288	190(65.9)	75	23	0	98(34)
Tetanus cases						
Infants	37	26(70.2)	9	2	0	11(29.7)
Neonates	73	56(76.7)	9	7	1	17(23.3)
Total	110	82(74.5)	18	9	1	28(25.5)

The antitoxin titres in sera samples of 23 out of 54 pairs of mothers and their neonates (Table 3) were less than 0.015 I.U./ml. In serum samples of eighteen mothers, antitoxin titres were equal to or greater than 0.015 I.U./ml while in 13 neonates they were >0.015 I.U./ml and in 41 neonates they were >0.015 I.U./ml. Antitoxin titres in mothers were 2–4 fold higher than in babies.

Normal control sera were found to be negative at the lowest dilution tested by toxin assay i.e. 0.5 ng/ml while 40% of all the tetanus cases showed toxin positivity (Table 4). The toxin positivity rates in neonates and infants were 36 and 59% respectively, showing a statistically significant difference ($\chi^2 = 55$, $p < 0.05$). Although toxin positivity rate was significantly higher in infants as compared to neonates, the latter had much higher levels (500–3000 ng/ml) of circulating toxin than the infants.

The antitoxin levels of sera samples from convalescent cases were less than 0.015 I.U./ml in nine cases and 0.5 I.U./ml in two cases. In three convalescent serum samples, an over four-fold increase of tetanus antitoxin level was observed and in one sample a two-fold increase was observed i.e. 0.06–0.125 (Table 5). The toxin levels of paired serum from 9 cases were below the lowest dilution tested and in three cases, the levels remained the same. In three other cases, an 8–12 fold drop in the toxin levels was observed in the convalescent sera samples.

The data were further analysed to investigate the relationship between the levels of toxin, antitoxin and the clinical outcome of the cases (Table 6). Out of 23 toxin positive cases in infants, 17 died and 6 recovered, whereas in 16 toxin negative cases 8 each died and recovered. This observed difference in the outcome of toxin positive and negative cases was not statistically significant ($\chi^2 = 2.33$, $p > 0.05$). In the antitoxin positive group, 7 died and 4 recovered, while in the antitoxin negative group, 11 died and 15 recovered. This difference was not found statistically significant ($\chi^2 = 1.35$, $p > 0.05$).

The same data, when analysed only in the neonatal cases (Table 7), showed a more interesting result. Out of 45 toxin positive cases, 12 (26.6%) died whereas out of 80 toxin negative cases, 23 (28.7%) died and 57 (71.2%) survived. The difference in the outcome between toxin positive and negative cases was statistically significant ($\chi^2 = 4.63$, $p < 0.05$), but the observed difference in the outcome between the antitoxin positive and negative groups of neonates was not statistically significant ($\chi^2 = 1.02$, $p > 0.05$).

The relationship of antitoxin titre with day of clinical illness was studied in 74 cases out of the total 110. In this group, sera samples were collected within 72 h of clinical onset of tetanus. It was found that 8 out of 40 cases had antitoxin level ≥0.015 I.U./ml on the first day of clinical illness and in 2 cases, it was as high as 0.5 I.U., thus showing a range of 0.015–0.5 I.U./ml. Out of the 6 patients who were admitted seven or more days after the onset of illness, four had antitoxin level of 0.06 I.U./ml (data not shown).

In 8 cases, both toxin and antitoxin were found to be present (Table 8). It was seen that seven out of the eight cases had died and only one recovered.

Table 3
Level of tetanus antitoxin in paired mothers and their neonates sera ($n = 54$).

Level of tetanus antitoxin in mothers (I.U.)	Level of tetanus antitoxin in neonates (I.U.)						
	<0.015	0.015	0.03	0.06	0.125	0.25	0.5
<0.015	23	–	–	–	–	–	–
0.015	6	1	1	1	–	–	–
0.03	4	–	–	–	–	–	–
0.06	2	2	1	–	–	–	–
0.125	2	–	1	2	–	–	–
0.25	3	–	1	–	–	–	–
0.5	1	–	–	–	1	–	–
1.0 & above	–	–	–	1	–	–	1
Total (54)	41	3	4	4	1	–	1

4. Discussion

Passive haemagglutination test has been standardized in the laboratory against standard toxin neutralization test for estimating tetanus antitoxin. A strong correlation ($r = 0.9$) was observed between the two tests confirming earlier reports [10,11]. However, this finding contradicted the reports of some of the previous authors who found a lot of discrepancy in the titre determined by the two methods, and concluded that the PHA test, though unexpensive, rapid, and useful in determining tetanus antitoxin in sero-surveys, may not be appropriate for analysing the antitoxin response of an individual [5,12]. The strong correlation observed between the two tests in the present study advocate the use of PHA test for determining antitoxin in tetanus cases.

The presence of a protective level of antitoxin in a sizeable proportion of a normal control group compared to tetanus cases could be attributed to antitoxin neutralization by the presence of toxin or vice versa. This finding was noteworthy particularly given the prevailing belief that natural immunization does not occur in tetanus. Our findings tend to support the observation of Veronesi et al. [13] and Wu et al. [14], who detected a protective level of antitoxin in a group of subjects who never received tetanus toxoid. National tetanus immunity was thought to be induced by sublethal doses of tetanus toxin or by fragments of toxin released from *C. tetani* located in the digestive tract as a result of ingesting tetanus spores [14]. However, the clinical implication of natural immunity still seems to be unresolved.

The presence of a protective level of antitoxin in the tetanus cases was a notable finding. It is not possible to have acquired this level of antitoxin through active infection as the majority had been admitted within 72 h. In the neonates this may be explained by the passively acquired antibody from the mother. However, despite the presence of a high level of antitoxin, these cases suffered from tetanus.

From the findings of antitoxin titration in 54 pairs of mothers and babies, it appears that in general the antitoxin levels of babies were lower than those of mothers who had high level of antitoxin because of high natural immunity due to the longer time for

Table 4
Level of tetanus toxin in normal and tetanus cases.

Group	No. tested	Toxin (ng/ml)						Total (%)
		<0.05	0.05–0.5	>0.5–5.0	>5.0–50	>50–500	>500–3000	
Normal control	50	50(100)	0	0	0	0	0	0(0)
Tetanus cases								
Infants	39	16(41)	4	2	13	0	4	23(59)
Neonates	125	80(64)	14	1	14	0	16	45(36)
Total	164	96(59.7)	18	3	27	0	20	68(40.3)

Table 5
Level of tetanus toxin and antitoxin in fifteen paired acute and convalescent sera.

No. of sera tested	Antitoxin I.U./ml		No. of cases	Toxin ng/ml	
	Acute	Convalescent		Acute	Convalescent
9	<0.015	<0.015	9	<0.05	<0.05
2	0.5	0.5	1	6	6
1	<0.015	0.06	1	3	3
2	0.015	0.06	1	12	12
1	0.060	0.125	2	12	<0.05
			1	12	1.5

Table 6
Outcome of circulating tetanus toxin/antitoxin in infants.

Toxin test	Outcome (%)		Antitoxin test	Outcome (%)	
	Died	Cured		Died	Cured
Positive (23)	17 (73.9)	6 (26)	Positive (11)	7 (63.6)	4 (36.3)
Negative (16)	8 (50)	8 (50)	Negative (26)	11 (42.3)	15 (57.6)
Total (39)	25 (64.1)	14 (35.8)	Total (37)	18 (48.6)	19 (51.3)

Table 7
Outcome of circulating tetanus toxin/antitoxin in neonatal cases.

Toxin test	Outcome (%)		Antitoxin test	Outcome (%)	
	Died	Cured		Died	Cured
Positive (45)	12 (26.6)	33 (73.3)	Positive (17)	4 (23.5)	13 (76.4)
Negative (80)	23 (28.7)	57 (71.2)	Negative (56)	13 (23.2)	43 (76.7)
Total (125)	35 (28.0)	90 (72.0)	Total (73)	17 (23.2)	56 (76.7)

Table 8
Outcome of patients with circulating tetanus toxin and antitoxin.

No. of cases	Age	Toxin (ng/ml)	Antitoxin (I.U./ml)	Outcome
1	7 days	3072	0.06	Expired
1	7 days	12	0.03	Expired
1	9 days	6	0.015	Expired
1	15 days	3	0.125	Expired
1	15 days	3	0.125	Expired
1	1 month	3072	2.0	Expired
1	2 month	12	0.06	Expired
1	12 month	3	0.06	Recovered

potential exposure compared to their babies. This may indicate that a higher antitoxin level in mothers is required for passive transfer. It should be mentioned that transplacental antitoxin acquisition may not play a decisive role in the resistance against the disease as in this study a sizeable proportion of neonates having the accepted protective level of antitoxin were suffering from the disease. All these observations tend to support the earlier findings of Mya et al. [15], that individual antitoxin level was not the sole criterion of immunity against tetanus.

The absence of tetanus toxin in the normal controls was expected. Tetanus toxin could be determined in only 40% of the tetanus cases. The absence of tetanus toxin in the remaining cases indicates that either the toxin was already fixed to CNS or neutralized by antitoxin. Alternatively, the toxin might have reached the CNS by routes other than the haematogenous one [16].

It is worth mentioning that convalescent sera showed a four-fold increase in antitoxin titre. The presence of a low level of toxin in the convalescent sample indicated that complete neutralization of toxin by antitoxin could not occur and would be eliminated over time.

The presence of toxin or antitoxin did not affect the outcome of the disease except in neonates where toxin positivity suggested bad prognosis.

Conflict of interest

Nil.

References

[1] Pawar AB, Kumavat AP, Bansal RK. Epidemiological study of tetanus cases admitted to a referral hospital in solapur. Ind J Community Med 2004;29(3):115–6.

[2] WHO India. Maternal & Neonatal Tetanus (MNT) elimination. Avaliable at: www.who.int/immunization monitoring/diseases/MNTE_initiatve.

[3] Ghosh, Sharma. Determination of tetanus and sepsis among the last neonatal deaths at household level in a peri-urban area of India. Postgrad Med J 2011;87:257–63.

[4] Murhekar MV, Bitrangunta S, Hutin Y, Chakravarty A, Sharma HJ, Gupta MD. Immunization coverage and immunity to diphtheria and tetanus among children in Hyderabad, India. J Infect 2009;58:191–6.

[5] Hardegree MC, Barile MF, Pittman ME, Maloney CJ, Schofield F, MacLennan R. Immunization against neonatal tetanus in New Guinea. a comparisons of tetanus antitoxin titre obtained by haemagglutination and toxin neutralization in mice. Bull WHO 1970;43:461–8.

[6] Ray SN, Ray K, Grover SS, Mohan M, Mishra BS, Sharma SP. Sero-conversion following DPT immunization. Indian J Med Res 1978;67(5):728–33.

[7] Barile MF, Hardegree MC, Pittman M. Immunization against neonatal tetanus in New Guinea. Bull WHO 1970;43:453–9.

[8] Maselle SY, Matre R, Mbise R, Hofstad T. Neonatal tetanus despite protective serum antitoxin concentration. FEMS Microbiol Immunol 1991;76:171–6.

[9] Homes RK, Perlow RB. Quantitative assay of diphtheria toxin and of immunologically cross reacting proteins by reverse passive haemagglutination. Infect Immu 1975;12:1392–400.

[10] Levine L, Wyman L. Tetanus immunity in the north and south. JAMA 1964;187(7):518–20.

[11] Chatterjee SC. A comparative study of the haemagglutination and bioassay procedure for the assay of guinea-pig anti-diphtheria and anti-tetanus sera. Ind J Med Res 1964;52:1241–9.

[12] Galazka AM. The immunological basis for immunization series. module 3: tetanus [document WHO/EPI/GEN/93.13]. Geneva: World Health Organization, www.who.int/vaccines-documents/DocsPDF-IBI-e/mod3_e.pdf; 1993 [accessed 07.12.04].

[13] Veronesi R, Cecin H, Correa A, Tavares J, Moraes C, Bertoldo OJ. New concepts on tetanus immunization naturally acquired immunity. J Hyg Epidmio Micro Immu 1975;19:126–34.

[14] Wu CJ, Ko HC, Lee HC, Tsai WC, Li MG, Pao YZ, et al. Decline of tetanus antitoxin level with age in Taiwan. J Formos Med Assoc 2009;108(5):395–401.

[15] Mya MM, To M, Sein SM, Win MT. Active immunization against tetanus III. result of a five year study. Southeast Asian J Trop Med Public Health 1980;11(4):543–6.

[16] Igoa MM, Calvo AC, Penas C, Manzano R, Olivan S, Munoz MJ, et al. Fragment C of tetanus toxin, more than a carrier. novel perspective in nonviral ALS gene therapy. J Mol Med 2010;88:297–308.