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Measurement of diphtheria and tetanus antitoxin in blood samples collected on filter paper disks

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SUMMARY

Large-scale surveys of immunity to vaccine-preventable diseases may be limited by the inconvenience and expense involved in collection of blood by venepuncture. An alternative method of collecting blood on filter paper for measurement of immunity to diphtheria and tetanus is described. The precut filter disks (Elisadiscs), originally developed for serological diagnosis of disease in pigs, have advantages over previously described methods in that they allow safe handling of minimal volumes of blood (5 μl) which can be conveniently quantified.

To compare values obtained by venepuncture and fingerprick, paired samples were collected from 60 subjects and diphtheria and tetanus antitoxin concentrations were measured by ELISA. There was no significant difference detected between samples collected by the two methods.

The results suggest that Elisadiscs are a reliable alternative to venepuncture for monitoring immunity to diphtheria and tetanus and would be useful for sample collection in remote areas and from children.

INTRODUCTION

Periodic surveys of immunity to vaccine-preventable diseases such as diphtheria and tetanus are important for monitoring the efficacy of immunization programmes and for identifying high-risk groups within a population. Until recent years, two major constraints limited the feasibility of conducting such surveys. Firstly, laborious and expensive *in vivo* neutralization assays were necessary if results were to be quantitative and reliable. Secondly, problems associated with collection of blood by venepuncture and storage of large numbers of specimens have led to the screening of convenience samples which may not be representative of certain populations.

The first limitation has largely been overcome by the development of *in vitro* tests such as ELISA, for measuring diphtheria and tetanus antitoxin in sera [1]. To address the second problem, alternative methods of blood collection have been described [2–6] and involve the use of filter paper for collecting microlitre quantities of blood at superficial skin puncture sites such as the finger, heel or earlobe. The small volumes of sera eluted from filter paper are adequate for *in vitro*

assays. One problem with the fingerprick method of blood collection is that quantitation of the volume of blood may be unreliable unless time-consuming assays are performed [2]. Another consideration is the safety of the handler during manipulation of blood-soaked filter papers. An improved method has been reported by Banks [5], who designed precut disks known as Elisadiscs for the serological diagnosis of Aujeszky's disease in pigs. The sensitivity of the Elisadisc procedure was found to be equal to that obtained by conventional methods of blood collection.

This paper describes the application of the Elisadisc method to measurement of diphtheria and tetanus antitoxin in humans.

MATERIALS AND METHODS

Subjects

Sixty adults aged 23–81 years, who were nursing staff members or clients of a private pathology clinic and nurse educators from a tertiary institution, participated in the study. Data were collected on the subjects' age, sex, self-reported vaccination history and preference for the method of blood collection.

Blood collection

Paired samples of blood were collected by venepuncture and fingerprick. Sera from venous blood were stored at 4 °C. Fingerprick blood was collected by piercing the skin with an Autolet device (Lancet Scientific and Surgical) and placing the underside of each lug of an Elisadisc [5] over the bleeding point until the upper surface appeared uniformly red. The Elisadisc consists of a 38 mm diameter central disk with six, 6 mm disks (lugs) attached at the periphery. The Elisadiscs used in this study were cut from Whatman No. 1 filter paper with a lead die cast by a printing firm. A central 6 mm hole in the Elisadisc enabled it to be threaded onto a glass rod for airdrying. Dried disks were stored at room temperature in labelled envelopes for up to 4 weeks. A single fingerprick generally provided sufficient blood to saturate at least 12 lugs (2 Elisadiscs).

Elution of blood from Elisadiscs

Dried lugs were cut from Elisadiscs and up to three lugs were placed in a U-shaped well of a microtitre plate (Nunc). Blood was eluted in 300 μl of phosphate buffered saline (PBS) pH 7·6, 0·05% Tween 20 (PBST) in a 2 h incubation at room temperature. The eluates were mixed by aspiration immediately before 100 μl were withdrawn and used directly in ELISA or diluted in PBS containing 5% skim milk powder.

To determine the average volume of whole blood per lug, known volumes of fresh blood were applied to multiple lugs and tested in parallel with (i) lugs saturated with unknown volumes and (ii) matched serum samples obtained by venepuncture. The Elisadiscs used in this study were calculated to contain 5 μl of whole blood per lug. Determination of antitoxin concentration assumed that the serum content of whole blood is 50%.

Antigens

Diphtheria toxoid (2600 Lf/ml; 2000 Lf/mg protein-N) and tetanus toxoid (1780 Lf/ml; 1200 Lf/mg protein-N) were obtained from the Commonwealth Serum Laboratories, Melbourne.

Standard sera

Human diphtheria and tetanus antitoxins (58 and 91 IU/ml respectively) were obtained from the Commonwealth Serum Laboratories, Perth. These reference sera were standardized against the International Standard by *in vivo* toxin neutralization assays.

Control serum samples for diphtheria and tetanus antitoxin (0·2 and 3·4 U/ml respectively, based on ELISA) were obtained from a volunteer blood donor.

ELISA

Microtitre plates (Linbro, Flow Laboratories) were coated overnight with 100 μl of either diphtheria toxoid (10·8 Lf/ml) or tetanus toxoid (1·78 Lf/ml) in 0·1 M sodium carbonate–bicarbonate buffer, pH 9·6. The coated plate was washed six times with PBST and blocked with 200 μl PBS containing 5% skim milk powder for 30 min at 37 °C. The wells were washed again and 100 μl of undiluted or diluted sera and eluates were applied in duplicate. Each plate contained serial dilutions of human diphtheria and tetanus antitoxins from which a standard curve was constructed. A control serum sample was included in each assay, to monitor inter-assay reliability. After 2 h at room temperature and six washes, 100 μl of goat anti-human IgG-alkaline phosphatase conjugate (Tago) diluted 1:2000 were added to each well. The plates were incubated for 2 h at room temperature and washed six times. *P*-nitrophenol phosphate (Sigma) was added (100 μl per well) and the assay terminated after 20 min by addition of 100 μl 2 M NaOH. Plates were read at 492 nm by a Titertek Multiskan Reader (Flow Laboratories).

Statistical analysis

Data were analysed with the Minitab Version 7.2 statistical package. The Student's *t*-test for paired samples was used to compare $\log_{10}$antitoxin concentrations obtained from venepuncture and fingerprick blood. Analysis of immunity according to age and subject classification was carried out using the chi-square test with Yate's correction.

RESULTS

Methodological studies

Experiments were set up to determine the optimum conditions for recovery of antitoxin from Elisadiscs. Standard volumes of blood were applied to Elisadiscs and eluted under various conditions. Antitoxins were recovered with similar efficiency when eluted in microfuge tubes and microtitre wells. However, recovery declined when eluates were stored overnight at 4 °C prior to ELISA or when the

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Table 1. *Diphtheria and tetanus antitoxin concentrations in sera obtained by venepuncture and in fingerprick blood eluted from Elisadiscs* (n = *60*)

	Diphtheria antitoxin (U/ml)		Tetanus antitoxin (U/ml)	
	Venepuncture	Fingerprick	Venepuncture	Fingerprick
Mean	0·181	0·141	2·621	2·046
Minimum	0·006	0·012	0·002	0·000
Maximum	0·744	0·468	24·000	13·800

elution volume was increased to 0·5–1·0 ml. The thickness of filter paper was found to be important, as recovery from Whatman 3 mm paper was only 76% of the value for the matched venepuncture sample, while > 99% recovery was achieved from Whatman No. 1.

Demographic data

Paired samples of blood were collected by venepuncture and fingerprick from 60 adults aged between 23 and 81 years (mean = 46 years). Forty-two percent of the subjects were male and 58% were female. Forty-five percent of the subjects were health professionals (nurses and/or nurse educators) and 55% were clients attending a private clinic. Of the 60 subjects questioned after blood collection, 22 (37%) preferred the fingerprick method, 5 (8%) preferred venepuncture and 33 (55%) had no preference.

Levels of immunity to diphtheria and tetanus

Table 1 shows the mean and range of concentrations of diphtheria and tetanus antitoxin measured in venepuncture and fingerprick blood.

The results from both sampling methods show that the mean concentration of tetanus antitoxin is approximately 15 times higher than that for diphtheria antitoxin. The Wilcoxon–Mann–Whitney test showed no significant difference between males and females in their level of immunity to diphtheria ($Wx = 698$, $p > 0{\cdot}05$) and tetanus ($Wx = 790$, $p > 0{\cdot}05$).

Comparison of antitoxin concentrations between venepuncture and fingerprick samples

The antitoxin concentrations determined by the two sampling methods generally showed close agreement, with Pearson correlation coefficients of 0·816 and 0·913 for diphtheria and tetanus antitoxins respectively (Figs 1 and 2). Paired *t*-tests, performed on the $\log_{10}$ transformed values for diphtheria and tetanus antitoxins separately, showed that no significant difference could be detected between blood samples collected by the two methods ($t = -0{\cdot}91$, $p > 0{\cdot}05$; $t = -0{\cdot}63$, $p > 0{\cdot}05$ respectively). The *t*-test analysis, however, is likely to be biased by the samples with higher values which generally show good agreement. Discrepancies between the sampling methods were more frequently observed at antitoxin levels below 0·05 U/ml. This is more clearly demonstrated in Fig. 3 in which data from Figs 1 and 2 are replotted to display the magnitude of disagreement and the relative bias. Overall, the fingerprick sampling slightly

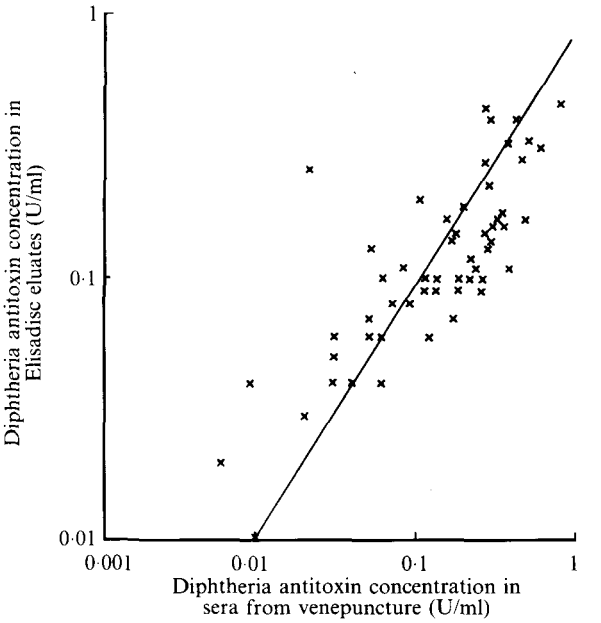

Fig. 1. Correlation between concentrations of diphtheria antitoxin measured in sera obtained by venepuncture and whole blood eluates from Elisadiscs.

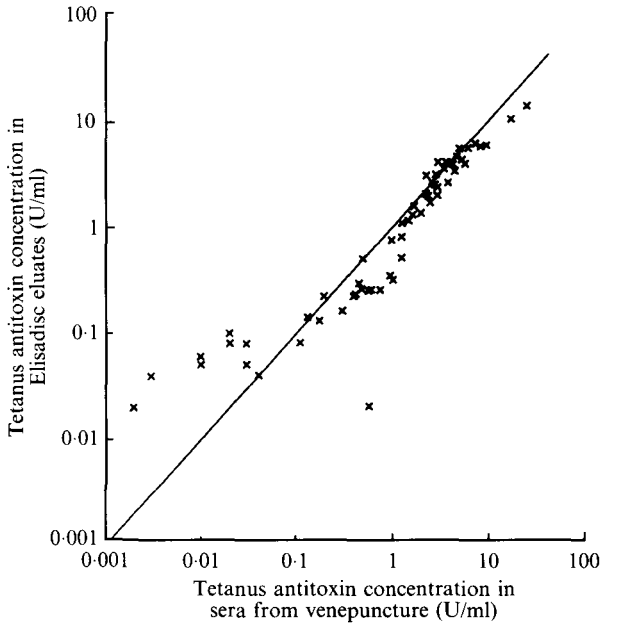

Fig. 2. Correlation between concentrations of tetanus antitoxin measured in sera obtained by venepuncture and whole blood eluates from Elisadiscs.

underestimates the values obtained by venepuncture, as indicated by the horizontal broken line corresponding to the mean of the differences between paired samples. For lower values, however, the fingerprick method overestimates antitoxin concentration, relative to the venepuncture method.

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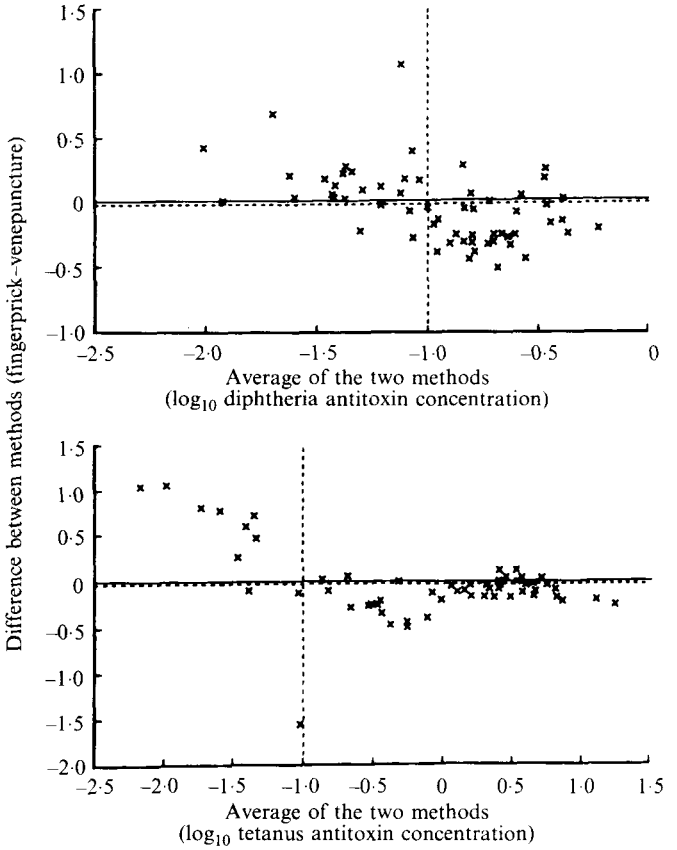

Fig. 3. Data from Figs. 1 and 2 replotted to display the magnitude of disagreement between the two methods of blood collection, for diphtheria (top) and tetanus (bottom) antitoxin concentration. The horizontal broken lines indicate the mean difference (−0·03) between the sampling methods, expressed as the $\log_{10}$ antitoxin concentration. The vertical broken line corresponds to the minimum protective level (0·1 U/ml) used in this study.

Table 2. *Number (%) of subjects protected versus not protected against diphtheria, according to method of blood collection*

	Venepuncture		
Fingerprick	Not protected	Protected	Total
Not protected	19 (32)	6 (10)	25 (42)
Protected	4 (6)	31 (52)	35 (58)
Total	23 (38)	37 (62)	60 (100)

Determination of the number of immune subjects

The antitoxin concentrations obtained by the two sampling methods were analysed to determine whether the method of blood collection influenced the number of subjects identified as protected, using 0·1 U/ml as the minimum protective level. Tables 2 and 3 show good agreement between the number of subjects classified as protected by each method. For diphtheria antitoxin, 37

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Table 3. *Number (%) of subjects protected versus not protected against tetanus, according to method of blood collection*

	Venepuncture		
Fingerprick	Not protected	Protected	Total
Not protected	9 (15)	2 (3)	11 (18)
Protected	1 (2)	48 (80)	49 (82)
Total	10 (17)	50 (83)	60 (100)

Table 4. *Number of protected subjects according to age group and subject classification*

	Number (%) protected	
	Diphtheria	Tetanus
Age group		
23–49 years ($n = 38$)	27 (71)	36 (95)
50–81 years ($n = 22$)	9 (41)	14 (64)
Subject classification		
Health professional ($n = 27$)	9 (63)	26 (96)
Client ($n = 33$)	19 (58)	25 (76)

(62%) venepuncture and 35 (58%) fingerprick samples were at or above 0·1 U/ml. For tetanus antitoxin 50 (83%) venepuncture and 49 (82%) fingerprick samples were at or above the protective level.

Analysis of immunity according to age was carried out after classification of venepuncture samples into two groups, 23–49 years (63%) and 50–81 years (37%). Table 4 shows that 71% of the subjects aged 23–49 years were protected against diphtheria, compared with 41% of the 50–81-year age group. This difference is statistically significant ($\chi^2 = 4{\cdot}09$, D.F. = 1, $p < 0{\cdot}05$). A significant difference between the two age groups was also observed for immunity to tetanus, with 95% of the 23–49-year group and 64% of the 50–81-year group classified as protected ($\chi^2 = 7{\cdot}59$, D.F. = , $p < 0{\cdot}01$). Comparison between health professionals and clients (Table 4) shows a higher incidence of protection against both diphtheria and tetanus in health professionals, but this difference is not statistically significant. Sixty-three percent of health professionals compared with 58% of clients were protected against diphtheria ($\chi^2 = 2{\cdot}6$, D.F. = 1, $p > 0{\cdot}05$) and 96% of health professionals compared with 76% of clients were protected against tetanus ($\chi^2 = 3{\cdot}4$, D.F. = 1, $p > 0{\cdot}05$).

DISCUSSION

Several studies have demonstrated the potential benefits of collecting blood samples on filter paper for surveys of immunity to diphtheria, tetanus [2, 6] and measles [4] and for serological testing for measles [7] and HIV infection [3]. Sample collection is simple and inexpensive. Dried filter disks occupy minimal storage space, do not require refrigeration and can be mailed in a standard envelope. Although long-term storage of filter paper disks was not tested in this study, previous reports state that dried discs may be stored at 4 °C or 25 °C for at least 12–18 months [5, 6].

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The major problem with the use of filter disks is the difficulty in quantifying the volume of blood collected. In some cases standard-sized disks have been cut from larger pieces of filter paper after blood collection and the serum content determined by measurement of the area of a near-circular spot of blood. This is then compared with a standard curve which correlates spot area and blood volume [2]. However, irregular spots are unacceptable and it has been observed that disks cut from the edge as opposed to the middle of blood-soaked areas give consistently higher values [5]. In addition, cutting out disks after blood collection is inconvenient and time-consuming. The precut Elisadisc [5] overcomes these problems and also facilitates safe handling as the blood-soaked lugs project away from the handlers' fingers. Multiple samples can be collected from a single fingerprick allowing for repeated tests if required.

This study has shown that diphtheria and tetanus antitoxin concentrations determined from fingerprick blood on Elisadiscs were closely correlated to venepuncture blood values.

Some discrepancy between the two sampling methods was observed at low antitoxin concentrations ($< 0{\cdot}05$ U/ml), but this may be related to limitations of the ELISA rather than the Elisadisc, since other studies have shown that in low-titre sera, antitoxin concentrations measured by ELISA tend to be overestimated and there is greater variability around the geometric mean [8, 9]. For this reason, some authors [8–10] recommend 0·1 U/ml as the minimum protective level for *in vitro* assays rather than the standard 0·01 IU/ml used for *in vivo* assays. The results of this study show that at the 0·1 U/ml minimum protective level, the fingerprick method is a reliable alternative to venepuncture for identifying those who are likely to be unprotected and require revaccination. Studies have suggested that it is prudent and safe to revaccinate at the minimum protective level [1, 8].

The assumption that a minimum ELISA value of 0·1 U/ml is required to confer protection can be misleading, however. A recent study by Sesardic and Corbel [11] found that 23% of subjects assessed for diphtheria antitoxin by ELISA, would be wrongly assumed not to need immunization against diphtheria. Other studies comparing *in vitro* immunoassays and *in vivo* bioassays for diphtheria antitoxin have also shown poor correlations [8, 12], indicating that the ELISA detects a high proportion of non-neutralizing antitoxin. Sesardic and Corbel [11] suggest that the best way to detect neutralizing diphtheria antitoxin is by a functional toxin neutralization assay, which has been shown to correlate well with the *in vivo* bioassay [13]. In the case of tetanus antitoxin there is generally good agreement between toxin neutralization tests in mice and ELISA [11] or another *in vitro* immunoassay, the toxin binding inhibition test [14]. Thus, protection against tetanus can be reliably determined by *in vitro* immunoassay.

In this study the high proportion of subjects protected against tetanus (83%) may reflect the number of younger subjects (63% were aged 23–49 years) and the number of health professionals in the study group (45%). Health professionals would be more likely to have received boosters due to employment requirements or increased awareness of the need for revaccination. However, the difference between the number of health professionals protected against tetanus (96%) and diphtheria (63%) suggests that revaccination against diphtheria may be perceived

as less important or unnecessary. An attempt was made to obtain self-reported vaccination histories from all subjects. However, since 38% of the total sample could not recall whether they had been vaccinated or received boosters, no attempt was made to compare vaccination history with levels of immunity. It was observed that 11% of health professionals could not recall whether boosters they had received within the last few years were for combined diphtheria and tetanus or tetanus alone.

Although the number of notified cases of diphtheria and tetanus in Australia has reached an insignificant level (8 cases of diphtheria and 7 cases of tetanus in 1991 [15]), the potential for diphtheria to re-emerge is illustrated by the 1984 outbreak of this disease in Sweden which had not recorded any indigenous cases of diphtheria from 1960 to 1983 [16]. In the case of tetanus, studies in developed countries have shown that the disease mainly affects the elderly who have either never been vaccinated or whose immunity has waned [1]. Thus, with the increasing proportion of elderly people in our communities, there is a need to encourage 10-yearly revaccination in adults (the recommended interval in Australia), and periodically to monitor vaccine uptake and levels of immunity.

The fingerprick method of screening with the Elisadisc is useful for large-scale surveys to gather information on general trends of immunity, particularly in remote areas, and to identify high-risk groups. The method is particularly suitable for children when evaluating B cell dysfunction and for monitoring responses to vaccination programmes.

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