Modelling anti-pertussis toxin IgG antibody decay following primary and preschool vaccination with an acellular pertussis vaccine in UK subjects using a modified oral fluid assay

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Recent vaccination with pertussis vaccine can confound serological and oral fluid (OF) assays targeting anti-pertussis toxin (anti-PT) IgG antibodies as a marker of recent infection. This study sought to establish the minimum potentially confounding time period based on experimental data to assist interpretation from such samples submitted from UK subjects for pertussis diagnosis. Anti-PT IgG antibody response and decay were measured post-vaccination using a modified OF IgG antibody-capture ELISA (GACELISA). Data were obtained from 72 infants after the third acellular pertussis vaccine dose in the primary schedule (4 months of age) and from 119 children after the single dose at preschool age (3 years 4 months to 5 years 8 months of age). Specimens were taken at approximately 1 month intervals for 9 months post-primary immunization (third dose) and 13 months post-preschool booster (PSB). The modified GACELISA demonstrated a sensitivity of 52/56 (92.9 %: 95 % CI 82.7–98.0) and a specificity of 120/128 (93.8 %: 95 % CI 88.0–97.3) and showed good agreement with the National Reference Laboratory standard anti-PT IgG serum ELISA (rank correlation = 0.80) and the original OF assay (rank correlation = 0.79). Modelling of the decline in antibody titres showed a reduction of 54 % and 34 % for each doubling of time after day 14 for the post-third primary dose and post-PSB subjects, respectively. These data suggest that the minimum confounding time period is approximately 300 days for samples obtained post-primary immunization and at least 3 years for samples submitted from UK children following immunization with the PSB. These data will greatly assist the interpretation of single high diagnostic anti-PT IgG titres by allowing an estimate of the positive predictive value, when the number of days post-immunization and prevalence are known or assumed.

INTRODUCTION

Laboratory methods for the confirmation of pertussis infection comprise, isolation of the causative organism, Bordetella pertussis, detection of its genomic DNA in clinical samples, typically upper respiratory specimens including nasopharyngeal aspirates and nasopharyngeal or pernasal swabs by PCR, and estimation of levels of specific antibodies to B. pertussis using serological or oral fluid (OF) assays (Fry et al., 2009; Guiso et al., 2011; He et al., 2012).

An IgG antibody-capture ELISA (GACELISA) to detect anti-pertussis toxin (anti-PT) IgG in OF for diagnosis and surveillance of B. pertussis infection in children and young adults was originally described by Litt et al. (2006). The assay detected seropositive subjects with a sensitivity of 79.7 % [95 % confidence interval (CI) 68.3–88.4] and a specificity of 96.6 % (95 % CI 91.5–99.1), using a cut-off of 70 arbitrary units (AU), compared to the standard serum
anti-PT IgG in the UK Pertussis National Reference Laboratory (NRL) (Pebody et al., 2005; Guiso et al., 2011). In contrast to collection of serum from young children, which can be difficult and have low compliance, OF collection is well accepted amongst these subjects and their parents/guardians, and samples can be self-collected. OF samples are stable when posted to the laboratory at ambient temperature and following frozen storage for several years (Morris et al., 2002; Mortimer & Parry, 1988).

The OF assay of Litt et al. (2006) was made available from the UK NRL on a pilot basis from June 2007 to August 2009 to all clinically notified cases of pertussis whose infection was not laboratory confirmed using other methods (i.e. culture, serology or PCR) and results from this pilot made a significant contribution to UK surveillance data (Health Protection Agency, 2007a, b; Campbell et al., 2012).

It is known that recent vaccination with whole-cell or acellular pertussis vaccine (wP or aP) elicits IgG against PT and this can confound serological (and OF) assays targeting anti-PT IgG antibodies as a marker of recent infection (Guiso et al., 2011; Watanabe et al., 2006). From 1990, the primary UK pertussis vaccine schedule was wP at 2, 3, and 4 months of age and pertussis components have been included in the preschool booster (PSB) dose (3 years 4 months to 5 years of age) as diphtheria/tetanus /- or 5-component acellular pertussis (aP3 or aP5) vaccine since October 2001. In October 2004, the wP was replaced by an aP5 vaccine in the primary schedule (Campbell et al., 2012). In the absence of experimental UK data we have used 1 year as the allotted time period post-vaccination, during which serological and OF results would be considered as potentially confounded. In this study we sought to better establish this time period based on experimental data in order to assist interpretation of anti-PT IgG titres from such samples submitted for diagnosis from individuals vaccinated with PT-containing vaccine.

It is generally accepted that in order to obtain a valid result using the GACELISA format, a sufficient concentration of total IgG must be present in OF samples in order to saturate the capture antibody layer. Previous studies indicated that this was achieved by IgG concentrations greater than 0.75 μg ml⁻¹ (Litt et al., 2006). However, during the UK pilot study some submitted samples contained less than the amount required for saturation. Therefore, in this study we also measured the total IgG in each sample and statistically modelled the data to determine if a correction factor could be derived which would enable titre derivation from samples with less than 0.75 μg ml⁻¹ total IgG to yield a valid result. The assay used here was modified from that of Litt et al. (2006).

The aims of this study were to: (i) deploy the modified OF GACELISA in order to measure anti-PT IgG response and decay in UK subjects following primary and PSB vaccination with pertussis-containing vaccine and (ii) model the decline in anti-PT IgG antibody titres to assist interpretation of results from such samples sent to the laboratory for diagnostic purposes.

METHODS

Study subjects and samples. One hundred and eighty-four matched OF and serum samples from patients who had been coughing for at least 14 days were used to establish the level of correlation between the modified OF assay and the NRL serum anti-PT IgG assay. These were taken from children and adults collected during a previous study (Harnden et al., 2006) and the UK pilot using the original OF assay to follow-up notified pertussis cases (Campbell et al., 2012).

Samples for the post-primary and post-preschool immunization cohorts were collected via seven general practitioner surgeries in Oxfordshire, UK, with informed consent following approval of the Oxfordshire research ethics committee. The sampling period was from July 2008 to December 2010. OF samples were taken from 169 infants and 138 children recruited to assess antibody response post-primary and post-PSB immunization, respectively. Samples were collected monthly from each subject for up to 9 months for the post-primary cohort and up to 13 months for the post-PSB cohort. The first swab for the primary cohort was taken on the day that the third primary immunization was administered (i.e. at 4 months of age) and for the preschool cohort as soon as possible after administration of the PSB. Age of preschool cohort at recruitment was 3 years 4 months to 5 years 8 months (mean, 3 years 7 months; median, 3 years 6 months).

OF was collected using an Oracol swab (Malvern Medical Developments) by rubbing the swab around the gum line for at least 1 min. The swab was then sent to the UK NRL by mail in suitable packaging. On receipt, OF was eluted from each swab, as described previously (Sheppard et al., 2001; Litt et al., 2006), with the following additional step: eluted samples were subjected to centrifugation at 10 000 g for 1 min in a 2 ml microfuge tube to allow removal of any particulate matter. Supernatants were carefully transferred into fresh microfuge tubes and retained and pellets were discarded. Eluted samples were then stored at −20 °C until required.

Pertussis toxoid vaccine information. At 2, 3, and 4 months of age, a single injection of vaccine is given containing, diphtheria, tetanus, pertussis, polio and Haemophilus influenzae type b (HiB) (dTaP/IPV/HiB), Pediaflex (Sanofi Pasteur MSD) which contains 20 μg pertussis toxoid. At 3 years 4 months to 5 years a single injection of vaccine is given containing, diphtheria, tetanus, pertussis and polio (dTaP/IPV or dTaP/IPV) (Repevax, Sanofi Pasteur MSD or Infanrix-IPV, GlaxoSmithKline). Repevax contains 2.5 μg pertussis toxoid and Infanrix-IPV contains 25 μg pertussis toxoid.

Generation of anti-PT mouse monoclonal antibodies and conjugation to horseradish peroxidase.

Monoclonal antibodies. BALB/c mice were immunized subcutaneously with 38 μg of B. pertussis toxoid, National Institute for Biological Standards and Controls (NIBSC), UK in saline/PBS containing 100 μg of N-acetyl muramyl-L-alanyl-d-isoglutamine hydrate (Sigma-Aldrich) and an equal volume of Freund’s incomplete adjuvant (Sigma-Aldrich) (total volume=0.2–0.3 ml per mouse) and boosted twice, on days 14 and 35 after primary immunization. Test bleeds were taken 10 days after the third immunization and sera tested for reactivity to PT (Sigma-Aldrich) by indirect enzyme immunoassay. One mouse received intravenous inoculation with 20 μg pertussis toxoid in saline on day 46 of the immunization schedule. Splenocytes from the immunized mouse were fused with the NS1 mouse myeloma cell line and hybridomas generated by standard techniques (Köhler & Milstein, 1975). Hybridomas secreting B.
pertussis-specific antibodies were identified by indirect ELISA using PT-coated microtitre wells and selected hybridomas were cloned twice by limiting dilution.

**Isotyping of monoclonal antibodies.** Goat anti-mouse IgG1, IgG2a, IgG2b, IgG2c, IgG3 and IgM immunoglobulins (Jackson ImmunoResearch Laboratories) were used for isotyping the generated mAbs by indirect ELISA. Hybridoma culture supernatants (100 μl) diluted 1:4 in PBS containing 0.05% v/v Tween 20 (PBST) were added to microwells coated with 0.25 μg ml⁻¹ PT and incubated for 1 h at 37 °C. Microwells were washed four times with PBST. Goat anti-mouse isotyping reagents were diluted 1/2000 in conjugate diluent (Microimmune) and 100 μl of each were added to the microwells and incubated for 1 h at 37 °C. The wells were washed as before and incubated with 100 μl of a 1/20 000 dilution of rabbit anti-goat IgG-horseradish peroxidase (HRP) conjugate (Sigma-Aldrich) for 1 h at 37 °C. Bound HRP conjugate was detected by colour development following addition of 100 μl per well of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Microimmune). Colour development was terminated by addition of 100 μl per well 0.5 M HCl and resultant optical densities measured at OD₄₅₀ and OD₆₅₀.

**Characterization of monoclonal antibodies by SDS-PAGE and Western blotting.** Bordetella pertussis toxin (Sigma-Aldrich) aliquots were electrophoresed under reducing conditions on a NuPAGE 12 % Bistris gel (Invitrogen) in NuPAGE MES SDS running buffer (Invitrogen), at 200 V for 50 min. The separated proteins were transferred onto nitrocellulose membranes essentially as described by (Towbin et al., 1979) and according to the manufacturer’s instructions (Invitrogen). Nitrocellulose membranes were incubated with supernatant from cloned mAb-producing hybridomas, diluted 1/20 in 1% w/v skimmed milk powder in PBST, or with a 1:500 dilution of serum from the mouse test bleed, for 1 h at room temperature. Membranes were then washed and incubated with a 1/20 000 dilution of rabbit anti-mouse IgG-alkaline phosphatase conjugate (Stratech Scientific). After washing, the bound IgG was detected with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as substrate (Leary et al., 1983).

**Anti-B. pertussis toxoid–HRP conjugate.** IgG antibody from one clone, 8H8, was purified on a protein A column and coupled to HRP by the method of Wilson & Nakane (1978).

**Modified capture ELISA for anti-PT IgG in OF.** The OF anti-PT IgG assay was modified from that described previously (Litt et al., 2006) and performed as follows. Unless otherwise stated, all steps contained 100 μl per well and each titre result was generated from the mean of duplicate wells. High binding polystyrene microtitre plates (cat. no. 767071, Greiner Bio-One) were coated at 4 °C for 3 h and washed once with PBST, then incubated with 240 μl per well of blocking solution (5% w/v Solupro/ Microimmune) for 2 h at 37 °C. The solution was then aspirated from the wells and the plates were dried for 18 h at 37 °C. Each plate was then sealed in a plastic pouch containing silica desiccant and stored at 4°C until required. Plates can be stored for up to 3 years, if kept dry at 4°C in sealed pouches containing desiccant.

On the day of testing, each precoated and blocked plate was allowed to equilibrate to room temperature. A lyophilized ampuole of World Health Organization International Standard Pertussis Antiserum (Human) NIBSC code: 06/140 (NIBSC) was reconstituted as recommended and then diluted 1:66 v/v in PBS containing 0.2% v/v Tween 20 and 10% v/v heat-inactivated fetal bovine serum (Invitrogen) (Diluent A). This dilution contained 10 μg ml⁻¹ total IgG (determined using an in-house assay, below) and was given the OF anti-PT titre of 1000 aU (modified assay arbitrary units). This was diluted in a twofold dilution series in an anti-PT IgG seronegative serum (also diluted in Diluent A to a concentration of 10 μg ml⁻¹ IgG) to generate a standard curve on each plate containing a consistent concentration of total IgG in each well. OF samples were thawed on the day of use and tested without further dilution. Standard curve and OF samples were added to the precoated plate, incubated at 37 °C for 1 h, and the plate was washed four times in PBST. Purified PT (Kaketsuku) was diluted to 0.5 μg ml⁻¹ in Antigen Diluent (Microimmune) containing 10% heat-inactivated fetal bovine serum and a 1/1000 dilution of a seronegative human plasma, added to the ELISA plate and incubated for 1 h at 37 °C. The plate was washed one final time and incubated with 3,3',5,5'-tetramethylbenzidine (TMB, Microimmune) for 10–15 min and the reaction stopped by addition of 100 μl 0.5 M HCl. Absorbance readings obtained at OD₄₅₀ were subtracted from those at OD₆₅₀ for each well using a Multiskan EX microtitre plate reader and Genesys software (Thermo Labsystems). The titre of PT-specific IgG was calculated (in aU) for each sample by comparison to a four-parameter standard curve fitted to the serially diluted standard serum.

**Quantification of total IgG.** The concentration of IgG in OF and serum samples was determined as previously described (Litt et al., 2006).

**Statistical methods.** Total IgG values of <0.1 μg ml⁻¹ were set to 0.1 μg ml⁻¹ for the antibody decay analysis. For the post-primary immunization dataset, results of >2.5 μg ml⁻¹ were set to a value of 5 μg ml⁻¹ and for the PSB results of >5 μg ml⁻¹ were set to 10 μg ml⁻¹. For the comparison of OF and serum anti-PT IgG titres, values of 0 and >1000 aU were given the values of 1 and 2000 aU, respectively. For the antibody decay analysis, anti-PT titres of >1000 aU and <3 aU were set to 1000 aU and 3 aU, respectively. For the post-primary vaccination response analysis, sequential OF samples were taken up to approximately 280 days following the third primary dose as well as on the date of the third dose, at 4 months of age (Day 0). For the PSB analysis, sequential OF samples were taken shortly after the dose (0–10 days) then at intervals up to about 380 days thereafter. Samples were excluded from analyses if a valid aU could not be obtained after duplicate testing.

The decline in antibody titres was modelled by taking log₁₀(aU) and performing a linear regression analysis of these values against time since vaccination, which was also on a log₁₀ scale, as this log-log relationship has been shown to provide a good model for antibody decline (Borrow et al., 2010).

Day 0 results were included using an indicator variable for this time point and decline was assumed to start occurring from day 14. An initial assessment of the effect of total IgG on titres in aU showed significantly reduced titres once total IgG was less than 0.5 μg ml⁻¹. Correction factors were therefore calculated which varied from increasing titres by about 1.6 fold if total IgG was 0.1 μg ml⁻¹ to 1.1-fold if total IgG was 0.4 μg ml⁻¹.

To account for outliers, which may be due to asymptomatic anti-PT IgG boosting following exposure to B. pertussis or infection, the model was also refitted after removal of results with large residuals (observed titre >10-fold greater than expected). The residual standard deviation was then used to obtain 95% prediction intervals for the modelled titres and to estimate the probability that a titre was >70 aU (the value used to indicate recent infection) by assuming a normal distribution for the log₁₀ titres with a mean equal to the modelled titre and standard deviation equal to the residual standard deviation. Finally, the positive predictive value (PPV) was estimated.
based on different true prevalence estimates (0.1 to 0.5), a sensitivity of 90\% and specificity of 1 (the modelled probability that the titre was \(\geq 70\) aU). The PPVs were then plotted against time.

**RESULTS AND DISCUSSION**

**Monoclonal antibodies to B. pertussis**

One hundred hybridomas were generated from the fusion experiment, of which 29 were initially reactive with \(B.\) pertussis toxin as demonstrated by ELISA. Four of these, all secreting antibodies of IgG1 isotype, were further characterized, one of which, 8H8, gave the highest signal to noise ratio in the ELISA, and consequently an HRP conjugate of this antibody was used in the modified anti-PT OF GACELISA described below.

**Modified anti-PT OF GACELISA**

Anti-PT IgG titres generated from OF using the modified assay were compared to serum titres from matched OF and serum samples collected from 184 subjects with prolonged coughs in order to assess the level of correlation (Fig. 1). Using the serum threshold of 70 international units (IU) anti-PT IgG ml\(^{-1}\) (70 IU ml\(^{-1}\)), the threshold above which is considered indicative of recent pertussis infection by the UK NRL, 56/184 (30\%) of the subjects were defined as seropositive. The OF and serum titres showed good agreement and had a rank correlation of 0.80. Selecting a threshold of 70 aU, the modified OF assay demonstrated a sensitivity of 52/56 (92.9\%: 95 \% CI 82.7–98.0) and a specificity of 120/128 (93.8\%: 95 \% CI 88.0–97.3) when used to determine seropositivity. Under these conditions, the assay would possess a PPV of 67–90\% as a marker of \(B.\) pertussis infection, for the published range of prevalence of 12–37\% in those with prolonged cough (Cherry, 1999; Litt et al., 2006). It would, similarly, show a negative predictive value of 99.0–96.0\% between these limits.

One hundred and fifty-three of the 184 matched oral fluid samples were tested in both the original and the modified OF assays. The results were compared and showed good agreement (93\%), based on a cut-off of 70 aU, and good correlation, rank correlation = 0.79 (data not shown).

In order to assess run-to-run variability of the modified assay, seven simulated OF (generated using the standard curve reagents) with titres ranging from 10 to 100 aU were each tested in up to 10 independent runs. The resulting mean titres across all runs possessed coefficient of variance (CV) values from 7.0 to 14.4\% (mean, 9.8\%).

**Post-primary immunization cohort**

Results fulfilling acceptance criteria were available for 72 infants who provided from three to nine sequential swabs, i.e. a total of 626 data points, with 68/72 (94\%) infants having 8 or 9 samples. Samples were taken from the day of the third post-primary immunization (Day 0) and up to 310 days thereafter. Results for the first 10 infants, as an indication of the variability between individuals, are shown in Fig. 2(a). Some individuals (not shown) had raised titres in the latter time points indicating possible asymptomatic boosting.

**Post-PSB cohort**

Results fulfilling acceptance criteria were available for 119 children from whom 7 to 13 sequential swabs were taken from 0 to 10 days (median 2 days) post-vaccination and up to 380 days thereafter (i.e. a total of 1253 data points). Most children, 105/119 (88\%) had 11, 12 or 13 swabs.
(median, 12). Results for the first 10 children, indicating the variability between individuals, are shown in Fig. 2(b).

**Estimation of total IgG concentration**

Total IgG concentrations in OF of $\geq 0.8 \, \mu g \, ml^{-1}$ were found in 342/625 (55%) of post-primary samples compared to total IgG concentrations of $\geq 0.8 \, \mu g \, ml^{-1}$ in 1245/1407 (88%) of post-PSB samples.

**Fitted model of anti-pertussis toxin IgG decline following primary and booster vaccination**

Fig. 3 shows the fitted models along with 95% prediction intervals. For the post-third primary dose (Fig. 3a) the relationship shows a reduction of 54% for each doubling of time (e.g. from 50 to 100 days). For post-PSB (Fig. 3b) the titres reached were similar but the reduction was slower at 34% per doubling of time.

**Modelling PPVs for post-primary and post-PSB titres**

Fig. 4 shows the estimated probability that a result exceeded the 70 aU threshold by time since vaccination and Fig. 5 shows the PPV at the 70 aU threshold for various true prevalence values and 90% sensitivity. As prevalence decreased from 0.5 to 0.3 to 0.1, a PPV of 90% was reached by 150, 210 and 320 days post-primary immunization but did not reach 90% post-PSB within 1 year, even at a prevalence of 0.5. The estimated PPV extrapolating to 1 year post-primary is 99%, 98% and 93% for prevalence values of 0.5, 0.3 and 0.1, respectively.

**Extrapolation of the model post-PSB**

For the post-PSB titres, extrapolation of the model past 400 days to 3 years allows estimation of the PPV at later time points (Fig. 6). Ideally more data are required to
validate this, but this extrapolation gives an estimated PPV at 3 years of 95 %, 89 % and 67 % for prevalence of 0.5, 0.3 and 0.1, respectively and the PPV reaches 90 % at 600 days if the prevalence is 0.5.

Concluding remarks

Modelling post-vaccination titres is useful for estimating the PPV of the assay for diagnostic purposes at different time points following vaccination. These data suggest that the use of 1 year post-vaccination as the potentially confounding time period before anti-PT IgG titres >70 IU ml$^{-1}$ or >70 aU can be interpreted as an indication of recent infection is valid following primary immunization. This does not mean that diagnostic titres above these thresholds cannot be interpreted at earlier time points, but the PPV of the assay at different times must be taken into account. Dalby et al. (2010) investigated antibody responses to PT after pertussis infection and in 20 subjects post-vaccination. Of the vaccinees, 13/20 fell to the Danish NRL threshold of 75 IU ml$^{-1}$ anti-PT IgG within 2 years, whilst five others were predicted to reach the cut-off in 2.8–6.6 years. The remaining two vaccinees had approximately horizontal decay profiles. We would concur with Dalby et al. (2010) that it is important to determine the (pertussis) vaccination status of individuals suspected of having pertussis when interpreting serological (or OF) results submitted for investigation. Many factors contribute to the host response to pertussis vaccination including age, number of immunizations and specific vaccine type received and/or previous pertussis infection. Unfortunately, information concerning which PSB vaccine

![Fig. 3. Modelled decline of anti-pertussis toxin IgG titres in arbitrary units (aU) from oral fluid samples showing corrected titres with 95 % prediction intervals. The 70 aU threshold is also shown (horizontal dashed line). (a) Post-third primary dose and (b) post-preschool booster.]
Fig. 4. Modelled probability of exceeding the 70 aU threshold by time since vaccination in days. (a) Post-third primary dose and (b) post-preschool booster.

Fig. 5. Modelled positive predictive value (PPV) at the 70 aU threshold by time since vaccination in days using three different prevalence values. (a) Post-third primary dose and (b) post-preschool booster.
(Repevax or Infanrix) was administered was not recorded. Thus, we are unable to determine if there was a difference (increase) in response to the 10-fold difference in pertussis toxoid contained in the Infanrix compared to Repevax. Whilst the vaccine type, regimen and anti-PT IgG threshold indicative of recent pertussis infection differ between the UK and Denmark, our data are in broad agreement with a minimum of 3 years as the time period post-vaccination with PSB after which single high anti-PT IgG titres may be used for serological or OF diagnosis of pertussis infection. Knowing the PPV by time since vaccination will also assist interpretation of titres from such assays in diagnosis.

Currently, there are no adolescent or adult pertussis-containing immunizations given as part of the UK routine schedule. From October 2012, pregnant women in the UK have been offered pertussis vaccination (Repevax), between their 28th and 38th week of pregnancy, to help protect their newborn babies. This was introduced as a temporary measure in response to the recent outbreak (see https://www.gov.uk/government/publications/whooping-cough-vaccination-programme-for-pregnant-women). In this study we modelled anti-pertussis toxin IgG antibody decay post-primary and preschool vaccination. Responses of adolescents and adults to pertussis-containing vaccines may differ and similar studies would help establish potential confounding periods in these older age groups.

In conclusion, the modified OF assay was successfully deployed in the measurement of anti-PT response and decay in UK infants and children post-vaccination. The modified assay showed good correlation with both the standard in-house serum anti-PT IgG ELISA and the original OF GACELISA. Currently, UK pertussis guidelines use 1 year as the time period post-vaccination, during which results from sample submitted for pertussis diagnosis would be considered as potentially confounded. Mathematical modelling of the decline in antibody titres in this study suggests that this time period is reasonable for samples obtained post-primary immunization. However, these data suggest that this time period should be extended significantly (to at least 3 years) for samples submitted from UK children following immunization with the PSB. Moreover, these data will greatly assist the interpretation of single high diagnostic anti-PT IgG titres by allowing an estimate of the PPV, when the number of days post-immunization and prevalence are known or assumed.

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