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Assessment of diagnostic accuracy of a commercial ELISA for the detection of *Toxoplasma gondii* infection in pigs compared with IFAT, TgSAG1-ELISA and Western blot, using a Bayesian latent class approach

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ABSTRACT

Serological methods are the most commonly used diagnostic tools to detect *Toxoplasma gondii* infections in pigs. In the absence of a readily available 'gold standard', an estimation of diagnostic accuracy is difficult to assess. A commercial ELISA (PrioCHECK® Toxoplasma Ab porcine ELISA, Prionics, Schlieren, Switzerland) for the diagnosis of *T. gondii* infection in pigs was evaluated in naturally infected animals from two distinct populations; indoor and outdoor living animals. An assessment of diagnostic accuracy, using a Bayesian latent class approach with adjustment for within indoor and outdoor farm clustering using random effects, was performed. Tests used for comparison were: IFAT; ELISA using native affinity-purified P30 (SAG1) *T. gondii* tachyzoite surface antigen (TgSAG1-ELISA); and Western blot with *T. gondii* tachyzoites lysate. The data set comprised 297 pig serum samples across outdoor ($n = 149$) and indoor ($n = 148$) farms in Argentina. The estimated sensitivity and specificity for the commercial ELISA were 98.9% (95% credible interval: 96.2; 100) and 92.7% (95% credible interval: 87.7; 96.6), respectively. The analysis of sera and plasma from pigs ($n = 6$) experimentally inoculated with 5,000 *T. gondii* oocysts revealed a pronounced antibody response beginning 2 weeks p.i. until the end of the observation period (11 weeks p.i.) in all animals. Meat juice obtained from inoculated animals after euthanasia also tested positive. These results suggest that the PrioCHECK® Toxoplasma Ab porcine ELISA may be a useful tool to perform serological diagnosis of *T. gondii* infections in pigs to control *Toxoplasma* infection in pigs and humans.

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1. Introduction

Toxoplasmosis is a globally distributed zoonosis and pork is considered one of the major sources for *Toxoplasma gondii* infections in humans. This parasite may also cause clinical disease in pigs and reproductive failure characterised by abortion, fetal mummification, stillbirth and neonatal mortality. In pigs severe clinical disease due to *T. gondii* seems to occur infrequently; however, several cases have been reported worldwide, mainly in neonatal and weaned piglets. The main clinical signs observed were anorexia, apathy, fever, cyanosis, dyspnoea, hind limb weakness and even death (Dubey and Beattie, 1988; Dubey, 2009a). Pigs are not routinely tested for *T. gondii* infection at slaughter because current meat inspection practices do not allow the detection of cysts due to their small size (generally less than 100 μm). Nevertheless, in recent years there has been an increasing interest in the *T. gondii* infection status of meat products (Kijlstra and Jongert, 2008).

The diagnosis of *T. gondii* infections in pigs can be accomplished by direct and indirect methods. Direct methods include microscopic examination of infected tissues, demonstration of *T. gondii* DNA in tissue samples and isolation of the parasite in experimental animal models. These methods are very specific but their sensitivity is limited by the size of the sample that can be analysed. In infected pigs, the parasite burden might be very low (one tissue cyst in 25–100 g of tissue) (Dubey, 2009b) and the chances of detecting parasite stages in the tissues by histopathological, immunhisto-

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chemical or molecular methods are limited. Some authors compared histopathology, bioassay and PCR for diagnosis of *T. gondii* infection in experimentally and/or naturally exposed pigs and reported a low diagnostic sensitivity for histopathology (Garcia et al., 2006) and PCR techniques (Garcia et al., 2006; Hill et al., 2006; Tsutsui et al., 2007). In contrast, bioassay in cats was proposed as a "gold standard" test to determine *T. gondii* infections (Gamble et al., 2005; Hill et al., 2006). The advantage of this method is that large amounts of tissues (500 g or more) can be tested by feeding to cats, with a positive result evidenced by shedding of oocysts in feces after 3–10 days. The sensitivity of the method is considered high as just a few bradyzoites are sufficient to trigger oocyst shedding (Dubey, 2001, 2006). The bioassay in mice is regarded as less sensitive (Dubey et al., 1995b); it may require a higher number of animals and it takes longer to obtain a result (seroconversion or detection of parasite multiplication). However, bioassays are generally not used for large-scale screening because they are expensive, time-consuming and not desirable from an animal ethics point of view. Moreover, bioassays may fail for different reasons, such as low parasite load, selection of tissues from infected animals where the parasite is not present or loss of parasite viability.

Indirect (serological) methods (e.g. IFAT, ELISA, modified agglutination test (MAT), Western blot (WB), latex agglutination test (LAT), and indirect hemagglutination test (IHA)) are based on the detection of antibodies against the parasite. These methods are generally highly sensitive and have been widely used for the diagnosis of *T. gondii* infection in pigs, but only a few studies evaluated serological methods against bioassay (Dubey et al., 1995a,b, 2002; Gamble et al., 2005; Hill et al., 2006; Gardner et al., 2010). To date, there are few commercial tests for diagnosis of *T. gondii* infection in pigs (Dubey, 2009b). A commercial ELISA (*Toxoplasma* Microwell Immunoassay Kit, Safe-Path Laboratory, Carlsbad, CA, USA) based on formalin-fixed whole *T. gondii* tachyzoite antigen was shown to have a high relative sensitivity when bioassay in cats was used as 'gold standard' (Hill et al., 2006). Serological tests appear to be the most feasible diagnostic approach for monitoring programs to determine the status of farms with regard to exposure to *T. gondii*, in order to implement control measures. However, the frequent lack of standardization of reagents and techniques, or validation of these tests, represents some of their disadvantages (EFSA, 2011). In the context of diagnostic test evaluation, typically no absolute 'gold standard', i.e. a diagnostic test having 100% specificity and 100% sensitivity, is available.

No 'gold standard' models (NGS) which use latent class approaches are increasingly used and are gaining general acceptance as a robust way of estimating diagnostic test accuracies in the absence of a true 'gold standard' (Enoe et al., 2000; Basanez et al., 2004; Branscum et al., 2005). If no true 'gold standard' exists, a comparison of new tests against an imperfect reference test might result in biased estimates for test accuracies of the new tests. Latent class analysis takes its name from the idea that the true disease status for each animal is only latently observed but can be "recovered" from the observed data through using an appropriate statistical model. The classical latent class analysis model in diagnostic testing, the Hui–Walter model, assumes conditional independence between test results given the true status of disease and constant sensitivities and specificities across different populations, which only differ with regard to their prevalence (Hui and Walter, 1980). The estimates are only meaningful if the assumptions are valid and, therefore, a careful justification both from a statistical and from a biological perspective of the assumptions made when fitting models to data, is essential (Joseph et al., 1995; Pepe and Janes, 2007). If conditional dependence between tests exists and is not dealt with appropriately in the model, then classification errors for both tests will be substantially underestimated when using the Hui–Walter model (Vacek, 1985).

Bayesian modelling has the ability to incorporate prior information as distinct from that contained in current study data. In parasitology or bacteriology, some authors have fixed the specificity of a diagnostic test based on the presence of the pathogen to be 1 (Dorny et al., 2004; Rapsch et al., 2006; Krecek et al., 2008), thus reducing the number of parameters that need to be estimated. The rationale behind this procedure is to assume that if a pathogen is detected, e.g. by microscopy, then the pathogen is actually present, thus no false-positives are possible or the specificity equals 100%. In other cases previous knowledge, e.g. from published diagnostic studies or expert opinions, have been used to derive specific prior information, which was then translated into specific beta-distributions (Branscum et al., 2005; Dabritz et al., 2007; Gari et al., 2008; Habib et al., 2008). The World Organisation for Animal Health (Office International des Epizooties, OIE) standard operating procedures (SOP) for validation and certification of diagnostic assays (http://web.oie.int/VCDA/eng/en_fichier_SOP.pdf) prescribes the use of Bayesian inference in addition to the validation of a diagnostic test in reference animals with a known disease status; also in animals with an unknown disease status; and states the need to give a clear rationale for the choice of specific priors.

In Bayesian latent class approaches, it is possible to fit a large number of models differing in their prior choices and covariance structure. The challenge then becomes to identify the model which best describes the information in the data (Spiegelhalter et al., 2002).

The aim of this study was to robustly estimate the test accuracies of a new commercial test kit to diagnose *T. gondii* infections in pigs (PrioCHECK® Toxoplasma Ab porcine ELISA, Prionics, Schlieren, Switzerland).

2. Material and methods

2.1. Experimental infection of pigs with T. gondii

2.1.1. Parasites

Experimental infections were performed with the CZ Tiger *T. gondii* isolate (type II) originally obtained by B. Koudela, University of Veterinary and Pharmaceutical Sciences, Czech Republic, from the feces of a captive Siberian tiger (*Panthera tigris altaica*) kept at the Dvůr Králové Zoo in the Czech Republic in 2005, and maintained since then by passages between mice and cats.

2.1.2. Animals

To obtain reference material (serum, plasma, meat juice) for the serological evaluation, nine weaned 4.5 week-old pigs born from sows that tested seronegative (IFAT titer <50) to *T. gondii* were purchased from an intensive pig production unit (Strickhof Lindau, Effretikon, Switzerland). All animals were housed indoors in a stable with a concrete floor and were fed commercial dry food, hay and water. Six of these pigs were inoculated orally with a suspension containing 5,000 *T. gondii* oocysts of the CZ-Tiger isolate in PBS. The three remaining pigs received only PBS and served as negative, non-infected controls. All nine animals were clinically monitored over 11 weeks and blood was taken from the right vena cava cranialis at weeks 0 (inoculation day), 1, 2, 3, 4, 6, 8 and 11 after inoculation in tubes with and without EDTA. Plasma and sera, respectively, were separated, aliquoted and stored at −20 °C until use. Rectal temperatures of the pigs was monitored daily for 4 weeks. All six inoculated pigs had fever (39.6–41 °C) between days 5 and 10 p.i. and one pig excreted soft feces, and was apathetic and anorexic from 7 to 8 days p.i. No further clinical abnor-

malities were observed in any animal during the study. The non-inoculated control animals remained within normal clinical parameters during the study. Eleven weeks after inoculation, all pigs were euthanised. Samples from hind limb muscles were frozen and thawed, and the generated meat juice was collected and preserved at −20 °C. All animal experiments were authorised by the Cantonal Veterinary Office of Zurich, Switzerland (permission No. 106/2010).

2.2. Field samples from pigs

Two hundred and ninety-seven serum samples from pigs were selected randomly from outdoor and indoor pig farms from four provinces in Argentina. The selected sera corresponded to 149 pigs from seven outdoor farms from Buenos Aires (six to 42 animals/farm) and from 148 pigs from 17 indoor farms from Buenos Aires, Cordoba, Santa Fe and La Rioja (two to 18 animals/farm). After collection, aliquots of the sera were preserved at −20 °C until use.

2.3. Serological tests

Field serum samples from pigs were tested for antibodies to *T. gondii* by IFAT, WB, TgSAG1-ELISA and by a commercial ELISA test designed specifically for pigs: PrioCHECK® Toxoplasma Ab porcine ELISA. In all tests, known negative and positive sera were included. The positive sera were obtained from the experimentally infected animals.

2.3.1. Commercial ELISA

The PrioCHECK® Toxoplasma Ab porcine ELISA (Lot No. TX100401M201; in the following referred to as PrioCHECK® Toxo ELISA) was performed according to the manufacturer's instructions. ELISA plates, precoated with cell culture-derived *T. gondii* tachyzoite antigen are provided with the kit. Serum and plasma samples were tested at a dilution of 1:50, and meat juice samples at 1:10. O.D. was measured at 450 nm (reference filter 620 nm) and the test results were normalised and interpreted by calculating, for each sample, a percentage of positivity (PP) value relative to the O.D. of the positive control (PP Sample = O.D. 450 nm Sample/O.D. 450 nm Positive Control × 100). A PP ⩾ 15 was regarded as positive and PP values below 15 were considered negative, as suggested by the manufacturer.

2.3.2. IFAT

Tachyzoites of *T. gondii* RH strain grown in VERO cells were used as antigen and rabbit anti-pig IgG conjugated with FITC (Sigma, Saint Louis, USA) at a 1:100 dilution in PBS pH 7.2 was employed as secondary antibody. Sera were diluted twofold from 1:50 and a titer ⩾ 50 was considered positive (Pardini et al., 2012).

2.3.3. Western blot

For WB, samples containing 2×10^7 cell culture-derived *T. gondii* RH strain tachyzoites were processed under non-reducing conditions as described previously (Pardini et al., 2012). Tachyzoite antigen bands corresponding to 22, 30 and 42 kDa (Chavez-Velasquez et al., 2005; Garcia et al., 2008) were selected to define positivity of the field samples. Sera were considered positive when reactivity to the 30 kDa antigen and at least one of the other selected bands was observed.

2.3.4. TgSAG1-ELISA

An ELISA using native affinity-purified P30 (SAG1) *T. gondii* tachyzoite surface antigen was performed as described previously (Pardini et al., 2012).

2.4. Statistical analysis

A Bayesian latent class approach was used to obtain estimates for test accuracies of the four serological tests using field serum samples. Since all four tests were based on antibody detection, conditional dependence between tests was considered. Two distinct populations (indoor and outdoor) were considered; within these populations, the prevalence on each individual farm was considered a random effect. Diffuse gamma distributions for the hyperparameters (1; 0.1) in the beta distribution for the random effects (shape) have been used in the model. Non-informative or flat priors using beta distributions (1, 1) were used for all sensitivities and specificities. Each of the 12 covariance terms between two tests was added separately. Model selection was performed by visually checking the two-way covariances and using the deviance information criterion (DIC), and by the effective number of parameters (pD) in the fitted model (Spiegelhalter et al., 2002). Lower DIC values were considered to be indicative of a better model fit. Different chains were run from different starting points to assess convergence to assure robust estimation; Gelman Rubin-statistics for convergence was examined. For each model, the first 20,000 iterations were discarded as burn-in and the next 100,000 iterations were used to parameterise the model. Models were fitted with the software JAGS (Just another Gibbs Sampler) (http://mcmc-jags.sourceforge.net/) version 2.2.0, the software R (http://www.r-project.org/) version 2.11.1 and the package coda (Plummer et al., 2006). The model code is available from the authors upon request.

3. Results

3.1. Experimental infection of pigs with T. gondii

When tested by PrioCHECK® Toxo ELISA, all experimentally inoculated pigs developed *T. gondii* antibody levels above the suggested threshold (PP ⩾ 15) in serum after 2 weeks p.i. (mean PP values 44.6 ± 16.7). The antibody levels remained above the threshold until the end of the study at 11 weeks p.i. (mean PP values 140.7 ± 3.4) (Fig. 1). No significant differences were observed when testing plasma samples. By analysis of meat juice samples by PrioCHECK® Toxo ELISA at euthanasia, PP values of 143.5 ± 3.7 were observed in the experimentally inoculated animals. Non-inoculated control animals remained seronegative during the whole study (PP values <3.1 in all serum, plasma and meat juice samples).

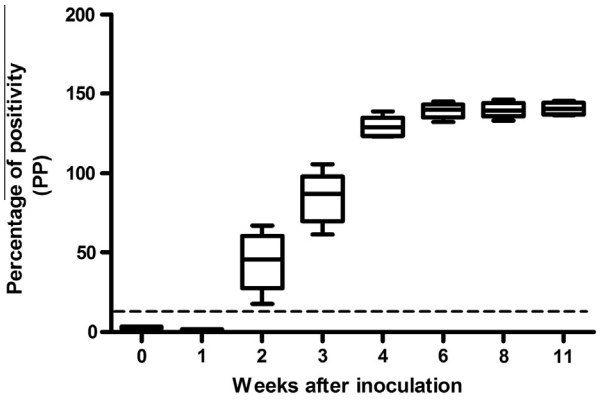

Fig. 1. Detection of antibodies against *Toxoplasma gondii* in sera of six pigs, experimentally inoculated with 5,000 *T. gondii* oocysts, by PrioCHECK® Toxo ELISA. Dashed line: percentage of positivity ⩾15 (cut-off).

3.2. Field study with four serological tests

The seroprevalences obtained using the four serological tests are presented in Table 1. The estimated test accuracies as posterior means and their corresponding 95% credible intervals resulting from the Bayesian latent class model are presented in Table 2, and the corresponding density distributions in Supplementary Figs. S1 and S2. The frequency of result combinations from the four serological tests within the outdoor and indoor pig populations are given in Table 3. Convergence of the final model, i.e. the robustness of the model's numeric estimates for test accuracies for all four serological tests is shown in Supplementary Fig. S3. The sensitivity and specificity estimates are given in the form of posterior density distributions (probability distributions), which are characteristic for the presentation of Bayesian latent class analyses. These probability distributions reflect what would occur if the field study was repeated on many samples of animals (sampling variability). As is standard in Bayesian practice, the three curves each represent a distinct simulation of the model. The curves are almost identical, thus indicating robustness of the model. Trace plots indicated sampling from a stationary distribution for all test accuracies as well as for shape parameters (available upon request). Convergence was also checked using the Gelman–Rubin statistics. Including two-way covariance terms between test sensitivities or specificities did not result in lower DIC values. Furthermore, adding two-way covariance terms did not result in different posterior estimates compared with the no covariance model. All histograms for each individual covariance term were strongly left-skewed against zero, indicating no evidence to support the inclusion of covariance terms between test accuracies of two tests (histograms available from the authors upon request). Therefore, the final model does not contain any covariance term. A table with DIC values for the different models is shown in Supplementary Table S1. The posterior estimates of the prevalences of the indoor and outdoor populations are shown in Supplementary Fig. S4. With the final model, the low prevalence estimates in the indoor population: 0.043 (95% (CI): 0.014; 0.085), in contrast to the high prevalence estimates in the outdoor population: 0.786 (95% CI: 0.65; 0.886), demonstrates the reliability of the model as it has correctly separated indoor and outdoor populations.

Table 1
Apparent prevalence of *Toxoplasma gondii* seropositive pigs in outdoor and indoor farms using four different serological tests (IFAT, Western blot, TgSAG1-ELISA and PrioCHECK® Toxoplasma Ab porcine ELISA).

Test	Apparent prevalence (%) of *T. gondii* seropositive pigs	
	Outdoor pigs (n = 149)	Indoor pigs (n = 148)
IFAT	80.5 (n = 120)	8.1 (n = 12)
Western blot	83.0 (n = 123)	23.0 (n = 34)
TgSAG1-ELISA	75.2 (n = 112)	4.7 (n = 7)
PrioCHECK® Toxo ELISA	83.2 (n = 124)	8.8 (n = 13)

Table 2
Comparison of diagnostic test accuracies of IFAT, Western blot, *Toxoplasma gondii* (Tg)SAG1-ELISA and PrioCHECK® *Toxoplasma* antibody porcine ELISA (PrioCHECK® ELISA) for detection of *T. gondii* infection in pigs, estimated using latent class analysis.

Test	Sensitivity (95% credible intervals)	Specificity (95% credible intervals)
IFAT	87.3 (80.9; 92.8)	87.0 (81.4; 91.8)
Western blot	93.5 (88.3; 97.4)	77.2 (70.4; 83.2)
TgSAG1-ELISA	93.1 (87.0; 97.8)	98.8 (96.6; 99.9)
PrioCHECK® ELISA	98.9 (96.2; 100)	92.7 (87.7; 96.6)

These results show that the PrioCHECK® ELISA is the most accurate test for detection of *T. gondii* infection in pigs in terms of diagnostic sensitivity, while the TgSAG1-ELISA is the most accurate test in terms of diagnostic specificity.

Table 3
Frequency of combinations of test results from four different serological tests (IFAT, Western blot, *Toxoplasma gondii* (Tg)SAG1-ELISA and PrioCHECK® *Toxoplasma* antibody porcine ELISA) within indoor and outdoor pig populations.

Western blot	PrioCHECK Toxo ELISA	TgSAG1-ELISA	IFAT	Indoor n = 148	Outdoor n = 149
–	–	–	–	102	8
–	–	–	+	5	8
–	–	+	–	0	0
–	–	+	+	0	0
–	+	–	–	6	1
–	+	–	+	1	4
–	+	+	–	0	1
–	+	+	+	0	4
+	–	–	–	24	6
+	–	–	+	3	3
+	–	+	–	1	0
+	–	+	+	0	0
+	+	–	–	0	5
+	+	–	+	0	2
+	+	+	–	3	8
+	+	+	+	3	99

4. Discussion

Pigs can be infected with *T. gondii* by ingestion of oocysts shed by felids or by eating tissue cysts from intermediate hosts and, less frequently, by transplacental transmission (Dubey and Beattie, 1988; Dubey, 2009b). During infection, antibodies against tachyzoites are produced regardless of the parasite stage that triggered the infection. The commercial ELISA kit uses a cell-cultured derived *T. gondii* tachyzoite antigen; therefore, we do not expect differences in its diagnostic properties depending on the infection source.

The prevalence of infection in pigs varies enormously according to the categories and age of pigs tested (e.g., piglets versus market pigs or sows) and the management system (e.g., free range versus bio secure indoor systems). Pigs from organic farms and free-ranging pigs often show higher prevalences as they have increased opportunities for contact with *T. gondii* (ingestion of oocysts, ingestion of infected rodents or birds) compared with animals reared indoors, under the assumption that hygiene and biosecurity measures are implemented in the latter situation (Venturini et al., 2004; van der Giessen et al., 2007; Dubey, 2009b; Davies, 2011; EFSA, 2011). When comparing surveys conducted up to three decades ago in the U.S.A. and in some European countries, a decline in the general seroprevalence of *T. gondii* infection in pigs is observed. However, the current trend in these countries of rearing pigs outdoors and in organic production may favor a rise in prevalence and enhance the significance of pork as a source of infection (van der Giessen et al., 2007; Dubey, 2009b; Davies, 2011). Interestingly, in a recent study from Switzerland, similar prevalences of *T. gondii* infection were observed in conventional fattening pigs and free-range pigs. This fact could possibly be attributed to the implementation of the new Swiss animal welfare regulations regarding animal-friendly housing systems, that gradually replaced the old conventional, hyper-hygienic closed intensive maintenance units guaranteeing, for example, access for the animals to straw or other materials high in fibre (Berger-Schoch et al., 2011). In our study, we selected two different pig populations from Argentina to perform an evaluation of the commercial ELISA test, where different prevalences of *T. gondii* infection could be expected

a priori; a high frequency of infection in the outdoor group and a low frequency in the bio-secure indoor group. Furthermore, the groups were built with pigs derived from different farms, in order to allow consideration of the frequency of infection in each farm as a random effect. Introducing random effects to account for correlated data is a standard approach to ensure valid statistical results.

We compared the results obtained with the commercial ELISA with those obtained by the other three serological methods using a Bayesian latent class approach in the absence of a 'gold standard'. As no true 'gold standard' was available and, strictly speaking, test accuracy is a population specific quantity, a latent class analysis was used. Latent-class statistical methods that do not require designation of a 'gold standard' are useful to estimate test accuracy (Hui and Walter, 1980) and, potentially, can prevent the bias that occurs in estimates of sensitivity and specificity if the test under evaluation is compared with an imperfect reference standard. The use of Bayesian inference has already been prescribed by the OIE in their standard operating procedures for test validation and certification of diagnostic assays (http://web.oie.int/VCDA/eng/en_fichier_SOP.pdf). In our study, we observed that the incorporation of any of the covariance terms in the 12 models did not affect the test accuracy and, based on DIC, the best model did not support conditional dependence between tests. By Bayesian analysis, all four serological tests evaluated in this study showed a reasonable to good sensitivity (87.3–98.9%); however, both ELISAs (PrioCHECK® Toxo ELISA and TgSAG-1 ELISA) showed a higher specificity (92.7% and 98.8%, respectively) than the IFAT and WB tests (87.0% and 77.2%, respectively). The different types of antigens used in each serological test and the operator-dependent subjectivity in the interpretation of results in IFAT and WB could account, to some extent, for these observations.

According to our results, the PrioCHECK® Toxo ELISA showed a high estimated sensitivity (98.9%) and specificity (92.7%), and might be a useful tool to perform serological diagnosis of *T. gondii* infections in pigs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2013.02.003.

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