

The stability of human, bovine and avian tuberculin purified protein derivative (PPD)

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Abstract

Introduction: Guidelines recommend storing tuberculin purified protein derivative (PPD) refrigerated. However, especially in developing countries, maintaining the product refrigerated under field conditions can be difficult, limiting its use. Here we determine the effect of prolonged exposure to high temperatures on the potency of human, bovine and avian tuberculin PPD.

Methodology: Human, bovine and avian tuberculin PPD were stored for several weeks exposed to temperatures ranging from 37° to 100°C. The potency was evaluated *in vivo*, in sensitized or naturally infected animals.

Results: Most test situations didn't affect the biological activity of the tuberculin PPDs and only very long and extreme incubations (several days at 100°C) compromised the potency.

Conclusions: Tuberculin PPD is very stable and can be stored or transported for long periods without refrigeration.

Key words: tuberculin PPD; stability; potency

J Infect Dev Ctries 2011; 5(11):781-785.

(Received 26 October 2010 – Accepted 28 June 2011)

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Introduction

New methods such as the QuantiFERON-TB Gold (QFT-G) assay (Cellestis Ltd., Victoria, Australia) and the T-SPOT.TB assay (Oxford Immunotec Ltd., Oxfordshire, UK) for humans and the Bovigam ELISA (Prionics AG, Zurich, Switzerland) for domestic cattle are available for the diagnosis of a tuberculosis infection. Still, the tuberculin skin test (TST) is one of the most used methods for the diagnosis of tuberculosis infection, particularly in developing countries, due to its relative low cost, easy applicability and minimal infrastructure requirements. To detect the pathogen in humans, a standard dose of 2 TU of Statens Serum Institute (SSI; Statens Serum Institute, Copenhagen, Denmark) tuberculin RT23 in 0.1 ml solution is injected intradermally and the diameter of induration across the forearm is measured 48 to 72 hours later. The result is recorded in millimeters and usually 10 mm or more of induration is considered positive. The diagnosis of tuberculosis infection in the subfamily Bovinae which includes domestic cattle, the bison and the water buffalo, is based on intradermal testing

with 0.1 ml of a solution of 1mg/ml (3.500-5.000 IU) of bovine tuberculin PPD. The increase in skin-fold thickness is measured with a calliper after 72 hours and a reaction is usually considered to be positive if there is an increase of 4 mm or more in thickness of the skin-fold. In addition, in some countries the comparative tuberculin test with bovine and avian tuberculin PPD is the official test for the detection of a *M. bovis* infection in cattle. The interpretation of this test is based on the comparison between the swellings caused by bovine and avian tuberculin after. When the greater reaction is produced by bovine tuberculin PPD, the disease is assumed to be of mammalian origin. However, a greater reaction at the site of injection of avian tuberculin PPD indicates that infection is due to either avian tuberculosis or Johne's disease (paratuberculosis). Additionally, avian tuberculin PPD is used for the diagnosis of tuberculosis in pigs and birds.

One of the limitations of the TST is the apparent instability of tuberculin to high temperatures. The current requirement for human tuberculin PPD RT 23, manufactured by the Statens Serum Institute, is

that it is to be stored and transported in a refrigerated state (2-8 °C) [1]. Manufacturers and international guidelines also recommend that bovine and avian tuberculin PPD be stored and transported in a refrigerated state [2]. These storage conditions suggest that the antigens present in tuberculin PPD are thermo-labile.

Maintaining the product under field conditions within this temperature range can be difficult and limits its use. For this reason, for the control of bovine tuberculosis in Ireland, the Irish Government demands that their suppliers package the avian and bovine tuberculin PPD side by side in the same box. It is considered that such pairing has practical benefits in field use but also theoretically ensures that the paired avian and bovine PPDs are subjected to identical conditions from the point of packaging to the point of use. In other words, one assumes that when the tuberculin PPDs are taken into the field (*e.g.*, in summer), they will lose their activity at the same rate but that this will have no influence on the test results when used in the comparative skin test. The supposed thermo-instability of tuberculin also leads to direct losses. In many countries the guidelines recommend discarding the tuberculin when exposed for a long period to temperatures above the recommended conditions. However, very few studies have addressed the stability of tuberculin PPDs when exposed to ambient temperatures for an extended period of time. The aim of the present study was to determine the effect on the potency of tuberculin when exposed for a long period to temperatures well above the recommended refrigerated range of 2-8°C.

Methodology

Human tuberculin PPD

We used 1.5 ml vials of PPD RT 23, 2TU/0.1 ml, lot No 1515K, expiry date 03-2010, for this study. Nine months before the official expiry date, vials of this lot were stored for varying periods at different temperatures; one vial at 37°C for 30 days (condition A); one vial boiled in a water bath for one hour in a vented bottle using a plugged syringe needle (condition B). At the conclusion of the respective treatments the tuberculin was stored at 4°C until potency testing in nine sensitized guinea pigs was completed against the control tuberculin PPD which had been kept at 4°C.

Avian and bovine tuberculin PPD

Five milliliter vials of Observe Bovine Tuberculin 32,500 IU per ml (batch 0902B, expire date June 2011) and Avian Tuberculin 25,000 IU per ml (batch 0801B expire date August 2010). Both the bovine and avian tuberculin were fromASUREquality Limited, Upper Hutt, New Zealand.

Vials of bovine tuberculin PPD were incubated at 37°C for three months (condition A) or boiled for one hour in a water bath in a vented bottle using a plugged syringe needle (condition B). At the conclusion of the respective treatments the tuberculin was stored at 4°C until potency testing in 22 naturally infected cows was completed against the control tuberculin PPD which had been kept at 4°C.

To determine the half-life time of both the bovine and avian tuberculin PPD, three vials of both PPDs were incubated for respectively 24 hours, one week and two weeks in an oven at 100°C. At the conclusion of the respective treatments, the tuberculin PPD was stored at 4°C until potency testing was completed in *M. avium* or *M. bovis* sensitized guinea pigs against the control tuberculin PPDs which had been stored at 4°C.

Tuberculosis skin test in guinea pig

The *in vivo* potency of human tuberculin PPD preparations was evaluated in nine guinea pigs which were previously sensitized with 500 micrograms dry weight of *Mycobacterium tuberculosis* strain H37Rv.

To determine the half-life time of the tuberculin PPDs, three guinea pigs were sensitized with 500 µg *M. bovis* strain AN5 and another three guinea pigs were sensitized with 500 µg *M. avium* strain D4.

The strains used to sensitize the guinea pigs had been heat-killed, freeze-dried and then suspended in liquid paraffin, previous to the intramuscular inoculation in the hind limb.

The guinea pigs were skin tested two to four months after sensitization with 0.1 ml of the control tuberculin maintained at 4°C and the respective heat-treated tuberculin PPD preparations. The test and control tuberculin PPDs were applied simultaneously to the same animal. The diameter of the skin reactions was measured in millimeters 24 hours after the application by a person who was uninformed about the different treatments of the tuberculins.

Tuberculin skin test in cattle

The *in vivo* potency of the control and treated bovine tuberculin PPD was evaluated in 22 Santa

Table 1. Evaluation *in vivo* of the stability of human tuberculin PPD RT 23 in guinea pigs infected with *M. tuberculosis* and bovine tuberculin PPD in cows naturally infected with *M. bovis*.

PPD RT23	Induration (mm)	SD	Bovine PPD	Mean skin-fold thickness increase (mm)	SD
(Control) PPD stored at 4°C	10.7	1.22	(Control) PPD stored at 4°C	6.6	2.85
(A) PPD stored at 37 °C for 30 days	10.0	1.93	(A) PPD stored at 37 °C for 30 days	6.7	2.46
(B) PPD boiled for one hour	10.3	1.32	(B) PPD boiled for one hour	6.4	2.77

The pre-treatment conditions (A and B) of the human and bovine tuberculin PPD are described in the methodology. The mean induration and the standard deviation (SD) of control and pre-treated PPD RT23, determined in nine sensitized guinea pigs, is given in columns 2 and 3 respectively. The mean skin-fold thickness increase and the SD of control and pre-treated bovine tuberculin PPD, determined in 22 cows, is given in columns 5 and 6 respectively.

Gertrudis cows. The cows were naturally infected with *M. bovis* and had been classified 6 weeks before as tuberculin positive by veterinarians of the National Bovine TB Control Program of the Ministry of Agriculture of the Bolivarian Republic of Venezuela. The tuberculin test was performed on the mid-neck; the injection site was clipped and cleansed and the thickness of a fold of the skin at the site of each injection was measured with callipers as accurately as possible in tenths of a millimetre both before and 72 hours after injection. Simultaneously 0.1 mL of the control and pre-treated tuberculins were injected in comparable sites about 10 cm apart. The increase in skin-fold thickness was recorded by the same person who measured the skin before and after the injection and was uninformed about the different treatments of the tuberculins.

Statistical analysis

The one way analysis of variance test (ANOVA) using the SPSS 14.0 (SPSS Inc, Chicago, IL, USA) software was used to evaluate the results

Results

Stability of Statens Serum Institute Tuberculin PPD RT 23

The results of the *in vivo* potency tests (in guinea pigs) of control and treated tuberculin PPD RT 23 preparations are summarized in Table 1. Tuberculin PPD stored at 37°C for 30 days or incubated for one hour at 100°C and control tuberculin PPD, which had been maintained at 4°C, showed no statistical difference when analyzed for indurations in sensitized guinea pigs (ANOVA $p = 0.961$).

Stability of Asurequality Bovine tuberculin PPD

The results of the testing of control and treated bovine tuberculin PPD in 23 naturally infected cattle are shown in Table 1. No significant difference in increase in skin-fold thickness was recorded with bovine tuberculin PPD stored at 4°C in comparison with tuberculin incubated for three months at 37°C or tuberculin incubated for one hour in boiling water (ANOVA $p = 0.902$). In figure 1 we show a cow with positive reactions for the control and pre-treated bovine tuberculin PPD preparations.

Half-life time of Asurequality Bovine and Avian tuberculin PPD

Because no “inactivation breakpoint” for the human and bovine tuberculin PPDs had been found in the previous experiments, vials of avian and bovine tuberculin PPD were incubated in an oven at a temperature of 100°C. After respectively 24 hours, one week and two weeks of incubation, a vial was taken out of the oven and stored at 4°C until used for skin testing. In figure 2 we show the results of skin testing of these tuberculin PPDs in sensitized guinea pigs. The results showed that 24 hours at 100°C had no significant effect on the potency of both tuberculin PPDs. The half-life time of the biological activity of avian and bovine tuberculin at this temperature was determined to be about one week.

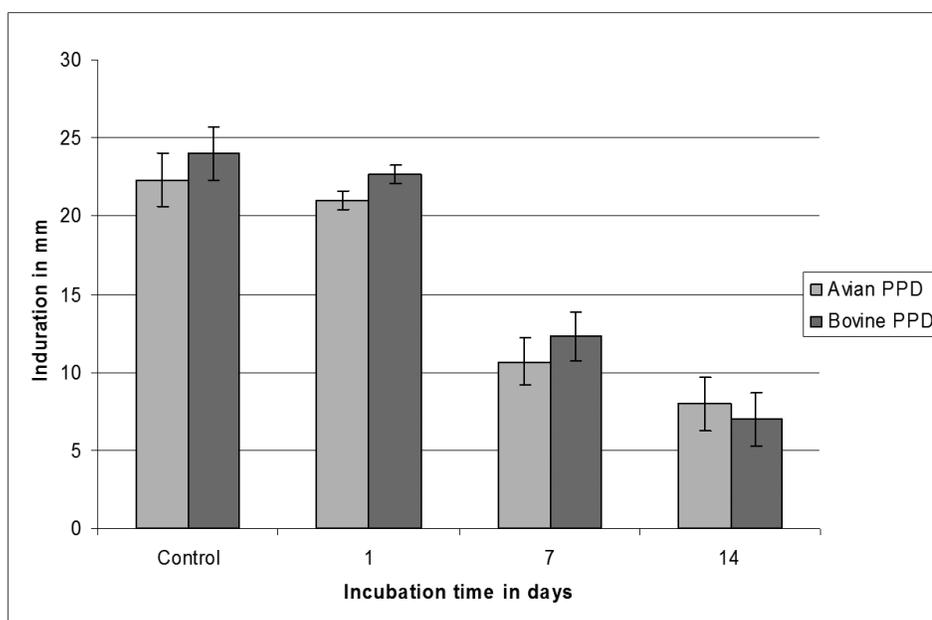
Discussion

The results of our study suggest that tuberculin PPD is thermostable. A boiling step of one to 24 hours does not affect the potency, demonstrating an important biological stability for this diagnostic reagent. This heat stability is in agreement with the production process for tuberculin which involves a heating step. In the production process of tuberculin

Figure 1. Mid-neck of a cow with positive reactions at three the injection sites after the application of tuberculin PPD stored at 4°C or incubated for three months at 37°C or one hour in boiling water respectively.



Figure 2. Determination of the potency breakpoint of bovine and avian tuberculin PPD. Vials of bovine and avian tuberculin PPD were incubated at 100 °C for 1 day and 1 and 2 weeks respectively and tested *in vivo* in guinea pigs against a vial of tuberculin kept at 4 °C (control) The mean indurations with the standard deviation (SD) in millimeters (mm) determined in 3 guinea pigs sensitized with *M. bovis* and 3 guinea pigs sensitized with *M. avium* are shown in the figure.



PPD 23 RT, the liquid *M. tuberculosis* cultures were sterilized by heating in “streaming steam” for one hour before proteins were precipitated and further purified [3]. The preparation of avian and bovine tuberculin PPD in general involves an autoclaving step of the *M. bovis* or *M. avium* culture at 121°C for 30 minutes [4]. In the production process of the avian and bovine tuberculin PPD employed in this study, Asurequality tuberculin PPD, the bacterial culture of *M. bovis* and *M. avium*, were sterilized in streaming steam for one hour (personal communication of the provider). These heating steps applied in the production process of tuberculin PPDs point toward a final product of denaturalized proteins, most probably thermo-stable.

Very few studies have addressed the temperature stability of tuberculin PPD. Landi *et al.*, investigating the stability of PPD-CT68 at different temperatures, have shown stability at high temperatures [5]. In their report, tuberculin PPD solutions of three strengths (1 TU, 5 TU and 250 TU per dose) were stable for at least three years at 4°C and for two years at room temperature (24 °C). At 37°C the solutions of all three strengths were stable for at least one year. As far as we know no previous studies evaluated the stability of human PPD RT23 or bovine and avian tuberculin PPD under high temperatures.

In this manuscript we show a significant temperature stability of tuberculin PPD. The tested human, bovine and avian tuberculin PPDs can be stored for at least 30 days without refrigeration at ambient temperature without losing potency. We are aware we have not tested all possible conditions, such as stability on repeated temperature cycling between refrigeration and ambient temperature, but our findings provide solid justification for people deviating on a case-by-case basis from the guidelines of storing tuberculin PPD at temperatures between 2-8°C. The stability of tuberculin PPD is of practical interest to medical and veterinarian personnel undertaking skin-testing in the field, permitting them to store, transport and use tuberculin without the requirement for refrigeration. This is especially important for the use of tuberculin PPD in developing countries, where cold chains (temperature-controlled supply chains, which, when unbroken, provide an uninterrupted series of storage and distribution activities to maintain a given temperature range that

help extend and ensure the shelf life of chemicals and pharmaceutical drugs) are often lacking. There is no longer the need to maintain temperature during transport of this biological, which presents a considerable saving on transport costs. In addition, we consider that there is no need to discard the tuberculin PPD when it has been exposed to temperatures above those recommended, as suggested by some TB programs. Of course, where practical and when the infrastructure is available, it would still be best practice for long-term storage to keep the diagnostic reagent at reduced temperatures but it is important to stress that the tested tuberculin PPDs resist temperatures far above the recommended storage temperatures of 2-8°C.

Acknowledgments

We are grateful to Veterinarian Dario Brillembourg and Ing. Lino Quiroz for technical assistance. MM, AD'A and FG can be considered as first authors as they developed and performed the special experiments with the human tuberculin PPD, the avian and the bovine tuberculin PPD respectively. Financial support was received from a LOCTI research grant from Shell Venezuela CA.

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Conflict of interests: No conflict of interests is declared.