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Quality control and diagnostic efficiency of bovine ppd (tuberculin) from indigenous *Mycobacterium bovis*

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ABSTRACT

Purified protein derivative (PPD) tuberculin was prepared from *Mycobacterium bovis* 3/86 and 4/86 strains isolated from Indian cattle. These *M. bovis* strains were harvested in bulk by growing them in Dorset Henley's liquid medium. PPD of *M. bovis* AN5 from Veterinary Laboratory Agency, Surrey, UK, was used as control. The PPD produced from *M. bovis* (3/86 and 4/86) were standardized by conforming to sterility, safety and potency in guinea pigs as per standard. All the 3 PPD preparations subjected to 12% polyacrylamide gel electrophoresis (SDS-PAGE) showed smearing along with 2 distinguished bands of 20–22 kDa. In 2-D electrophoresis, in first-dimension IEF by IPG strips, distinct banding patterns were not identified in PPD tuberculin's, in second dimension, there were distinct spots in acidic positions as well as in basic position in PPD *M. bovis* 3/86. DTH response with PPD *M. bovis* (3/86 and 4/86) was significant at 72h post-inoculation of tuberculin. There was no significant variation ($P<0.05$) of lympho-proliferative responses in PPD (*M. bovis* 3/86, 4/86) and PPD *M. bovis* AN5. A correlation between DTH response and lymphoproliferative response was found against different PPD tuberculins. Conclusively, our study indicated that *M. bovis* 3/86 would carry potential for large scale production of PPD and the tests applied may serve as quality control of PPD.

Key words: *Mycobacterium bovis*, PPD, Quality control, Tuberculin

This paper reports quality control and efficiency of indigenous purified protein derivative (PPD) in diagnosis of tuberculosis in animals.

MATERIALS AND METHODS

Experimental animals: Apparently healthy, 3-month-old male cattle calves obtained from the Division of Livestock Production Management, Indian Veterinary Research Institute (IVRI), Izatnagar; housed under conventional conditions in the animal shed, were provided green fodder, concentrate and water *ad lib.*

Apparently healthy, adult female guinea pigs (200–300 g) were obtained from the institute. The animals housed under conventional conditions in the animal shed, were provided green fodder, concentrate and water *ad lib.*

Mycobacteria for PPD production: M. bovis isolated from lymph nodes of cattle died with TB, identified and characterized with conventional tests as *M. bovis* 3/86 strain and *M. bovis* 4/86 strain, respectively, and maintained at Mycobacteria Laboratory, IVRI, Izatnagar, were used for production of PPD. These two strains were also confirmed for the presence of phenolic glycolipid (Dandapat *et al.* 1999) and with PCR assays of 500 bp and pncA gene (Shah *et al.* 2002).

Reference PPD: Bovine PPD AN5 (Lot 01, Product Code PA2022) produced at Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, UK, kindly provided by Dr Stephen Gordon, was used for *in vitro* comparison.

PPD production

Master seed: M. bovis 3/86 and 4/86 were passaged on Lowenstein Jensen medium with 0.5% Pyruvate (LJ-P) and were checked for purity by cultural and biochemical (niacin, pyrazinamide, nitrate, thio phen 2 carboxylic acid hydrazide) tests.

Production seed: M. bovis 3/86 and 4/86 used in the preparation of PPD (tuberculin) were freshly prepared from master seed (14-day old) by culture on 250 ml Dorset Henley's liquid medium, incubated at 37°C for 1 month. From these propagation flasks pellicles (approximately 6–8 mg) were seeded on to the surface of Dorset Henley's liquid medium for 12 weeks at 37°C and then liquid culture were steamed at 95–100°C for 3 h and cooled overnight. Later the culture was filtered through sterilized muslin-cloth; tuberculo-protein in culture filtrate was precipitated using

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40% trichloro acetic acid (TCA) (1 ml TCA + 9 ml filtrate). The siphoned-off supernatant was centrifuged to precipitate and dissolved with protein solvent fluid (minimal amount) and to it glucose buffer was added in equal amount. The protein content in the PPD (*M. bovis* 3/86 and 4/86) was estimated as described by Lowry *et al.* (1951). The total protein yield was calculated and expressed as mg per ml of PPD and finally the protein concentration was adjusted to 1 mg per ml.

Quality control of PPD

Sterility testing: 0.5 ml of PPD (*M. bovis* 3/86 and 4/86) was added into 20 ml fluid thioglycollate medium (FTM) and 20 ml soyabean-casein digest medium (SCDM) and incubated at 37°C for 14 days, and it was checked regularly for growth (Indian Pharmacopoeia 1996).

Safety testing: Two healthy guinea pigs, each weighing 200–300 g, that have not been treated previously with any material that will interfere with the test, were injected subcutaneously with 0.5 ml of the PPD (*M. bovis* 3/86 and 4/86), and monitored for 7 days for any abnormal effects. Two healthy guinea pigs, each weighing 200–300 g were injected 5 ml of the tuberculin intra-peritoneally and observed for 45 days, and these were examined macroscopically at post mortem (Indian Pharmacopoeia 1996).

Potency testing: The *M. bovis* AN5 bacilli were suspended in buffer and made into an emulsion with Freund's incomplete adjuvant. 0.5 ml of this suspension was inoculated intramuscularly into 8 healthy guinea pigs, weighing around 200–300 g for their sensitization. The potency test was carried out 4 weeks post sensitization (Indian Pharmacopoeia 1996).

The PPD tuberculin (*M. bovis* 3/86 and 4/86) were bio-assayed in sensitized guinea pig by an 8 point assay comprising 4 dilutions corresponding to 10 µg, 1 µg, 0.2 µg and 0.04 µg (dilutions were prepared using isotonic PBS (pH 6.5–7.5) containing 0.0005 g of polysorbate–80 R) 0.1 ml of each of PPD was inoculated intra-dermally into 8 sites employing a Latin square design in each guinea pig. The reading of the assay was taken 24 and 48 h after the injection of the tuberculin.

Electrophoretic analysis

SDS - PAGE analysis of PPD: The protein profile of PPD *M. bovis* (3/86 and 4/86) and PPD *M. bovis* AN5 (U.K) was analyzed using SDS-PAGE as per Laemmli (1970). A slab gel electrophoresis system with vertical medium size gel apparatus (12 cm × 13 cm) was used with 12% poly-acrylamide gel containing 0.1% of SDS.

Two-dimensional electrophoresis of PPD: 2D-PAGE of PPD *M. bovis* (3/86 and 4/86) and PPD *M. bovis* AN5 (UK) was carried out using the method of 'in gel rehydration' (Gorg *et al.* 2000). PPD mixed with rehydration buffer (final volume 125 µl) was applied to immobilized pH gradient (IPG strip) and was left overnight for passive rehydration at 20°C. IEF was performed in an IEF protean cell using the four step program. Step 1. 0–250 V for 1 h, linear mode; Step 2. 250 V constant for 1 h, rapid mode; Step 3. 250–3 000 V for 4 h, linear mode; Step 4. 3 000 V constant until 15 kVh, rapid mode. After IEF, IPG strips were equilibrated sequentially in equilibration buffer I and II. Later, strip was loaded on the top of a vertical SDS-PAGE (12% gel) and sealed in place with 1% agarose dissolved in electrode buffer. Molecular mass markers were loaded in a separated well by the side of the strip. Electrophoresis was performed as per Laemmli (1970).

Delayed-type hypersensitivity test (DTH)

One flask seeded with *M. bovis* AN5 (250 ml flask contain 100 ml culture of *M. bovis* AN5 growth (1.5-month-old) was steamed for 3 h at 100°C and than kept overnight at room temperature and decanted medium. 50 ml of left over medium with settled growth was mixed vigorously in shaker 3–4 times. 20 ml liquid paraffin was added and it was mixed thoroughly. Four out of 5 healthy, 7–9 month-old male cattle calves were sensitized by inoculating 3 ml of heat killed culture of *M. bovis* AN5 intramuscularly (I/m) in the thigh region.

DTH reaction against each PPD was observed in sensitized cattle calves at the day of sensitization and 45 days of post sensitization. The single intra-dermal test (SIDT) was performed in the cervical (neck) region of calves. An area in the middle of neck was shaved and 3 separate locations within this area were identified. The thickness of the skin at these three areas was measured using Vernier calipers and recorded. The test was performed in calves by injecting intra-dermally 0.1 ml (1 mg/ml) PPD 3/86 and 4/86, respectively at different identified locations. Skin thickness was measured after 24, 48 and 72 h. The differences between pre-inoculation and post-inoculation skin thickness were calculated for each injection site.

Lymphocyte transformation test (LTT): PBMC's were separated from density gradient centrifugation as described by Boyum (1968). Mononuclear cells were finally suspended in RPMI–1640 medium containing 10% fetal calf serum. The viability of cells was ascertained by 0.2% trypan blue dye exclusion method. Lymphocyte culture was performed as described by Anderson *et al.* (1991). The PBMC's separated from calves were suspended in RPMI–1640 growth medium containing 10% FCS, 25 mM HEPES and antibiotics (streptomycin 50 mg/ml and penicillin 100 IU/ml) to have a final cell concentration of 5×10^6 cells/ml. The 100 µl of this cell suspension was poured in each well of 96 well flat bottom tissue culture plate. Another 100 µl of medium containing 10 µl of different PPD [from *M. bovis* 3/86 and 4/86, and from *M. bovis* AN5] (1 mg/ml), and 10 µl Con A (0.2 mg/ml) as positive control and medium alone as negative control were added to make the final volume of each well up to 200 µl. The plates were maintained at 37°C with 5% CO_2

in a humidified chamber for 3 days.

Lympho-proliferative assay was performed by using MTT (3–[4, 5-dimethyl thiazol–2-yl]–2, 5 diphenyl tetrazolium bromide) dye reduction method (colorimetric assay) as described by Mosmann (1983). After 3 days of incubation of lymphocyte culture at 37°C, 100 µl of supernatant was removed from each well and 15 µl of MTT dye solution (5 mg/ml) was added. The plates were further incubated at 37°C for 4 h. In MTT assay, after 4 h of incubation, 150 µl of solubilization buffer or DMSO was added and plates were kept at room temperature for 20–30 min to dissolve the formazan crystals (Cooke and O'Kennedy 1999). The reading (OD value) was taken at 540 nm in an ELISA reader. Stimulation index (SI) was calculated.

Statistical analysis

The differences between the potency testing, DTH response and antigen stimulated lymphocyte proliferated responses were analyzed by student's 't-test' and ANOVA.

RESULTS AND DISCUSSION

The growth of each *M. bovis* strain was harvested after 3 months of incubation. Each harvest was checked for the purity of culture by Zeihl and Neelson (Z N) staining. The PPD from each flask were prepared separately by precipitating with TCA, dissolved in R–30 buffer and quantified. Although both of these strains grew well on Dorset Henley's liquid medium, however, *M. bovis* 3/86 yielded luxuriant growth. The final concentration of protein in each PPD was adjusted to 1 mg/ml with R–31 buffer and stored under refrigeration.

The finished products of PPD *M. bovis* 3/86 and 4/86 were standardized using sterility, safety and potency test. In sterility test, each PPD (from *M. bovis* 3/86 and 4/86) was found sterile. In safety test, no adverse affect or any gross lesions on post-mortem were noticed in intra-dermally and intra-peritoneally inoculated guinea pigs. Both PPD (from *M. bovis* 3/86 and 4/86) were found safe in guinea pigs. In potency test results were read after 24 h and 48 h of inoculation and expressed as diameter of the area of reddening in mm. PPDs (*M. bovis* 3/86 and 4/86) elicited approximately similar DTH reaction. The results showed an intense DTH reaction at 24 h than 48 h. The diameter of the skin reaction following intra-dermal injection of tuberculin into guinea pigs are linearly related to the logarithm of the dose injected (Long *et al.* 1954), which was also found in our study.

The protein profile of PPD *M. bovis* (3/86 and 4/86) and PPD *M. bovis* AN5 (UK), in different concentration (60 µg, 80 µg and 100 µg) were analyzed by SDS-PAGE using 12% polyacrylamide gel, which appeared as an undefined smear. Similar smearing of PPD tuberculins has also been reported (Tameni *et al.* 1998); the explanation for such a smearing may be due to the fact that PPD produced from 3 months incubated culture of *M. bovis* 3/86 and 4/86, which contained more than 3 100 distinct polypeptide (Belisle 2005). However, in our study all PPDs showed 2 distinct bands at the bottom of 12% polyacrylamide gel (20–22 KDa).

In one dimensional IEF by IPG strips, distinct banding patterns were not identified in all PPD tuberculins, in the framework of notable differences of protein content and, therefore, of staining intensity; most protein focused in acidic position, further more, a broad band was consistently found in acidic position. Our findings were in partial agreement with (Tameni *et al.* 1998) who also found proteins focused in acidic position in 2-D electrophoresis of fourth week of culture from the BCG and AN5 strain. In second dimension, we also found some distinct spots in acidic positions around 20–22 KDa (2 spots) and around 38–44 KDa (2 spots); and some distinct spots in basic positions around 45 KDa (1 spot) and around 60 KDa (1 spot) in PPD from *M. bovis* 3/86, but this distinct pattern was not found in all other PPDs even after repeated experiments. This finding was different from the findings of Tameni *et al.* (1998) that reported expression of mainly acidic components, and also reported a basic cluster of 20–30 kDa, only revealed in the BCG 4-week tuberculin. This difference could be due to the use of (i) different strain, (ii) incubation period, and (iii) differences in production protocol for producing the PPD. It is conjectured that probably 2-D electrophoresis may not be able to provide analysis of PPD manufactured from 3 months incubated *M. bovis* culture (Fig. 1).

Simulation of naturally occurring infection was a desired goal in establishing methods for assaying the biological potency of PPD. This was especially significant because it had been demonstrated that tuberculin skin responses were altered in cattle artificially sensitized with killed mycobacterium suspended in adjuvant (Huitema 1973). In this study, we have evaluated the DTH skin responses of PPD (*M. bovis* 3/86 and 4/86) in experimentally infected

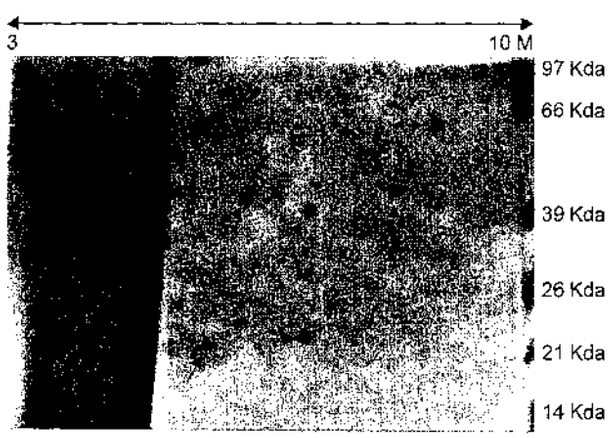
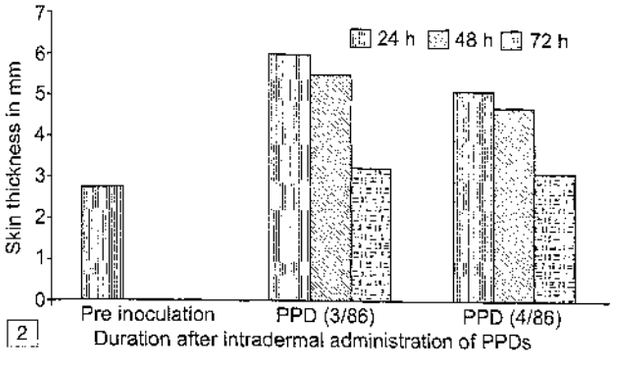
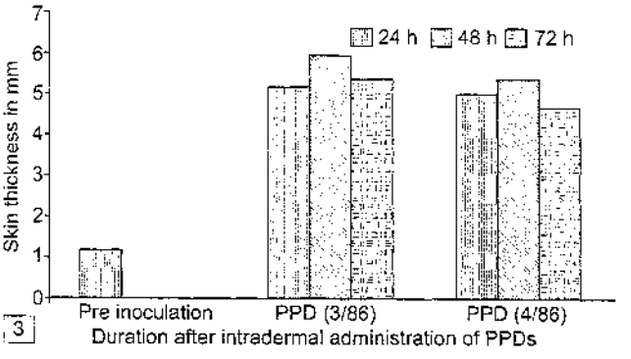


Fig. 1. 2-DE Pattern of PPD from *M. bovis* 3/86 on the IPG strip of pH range 3–10 (in first dimension) and on 12% polyacrylamide gel (in 2nd dimension).

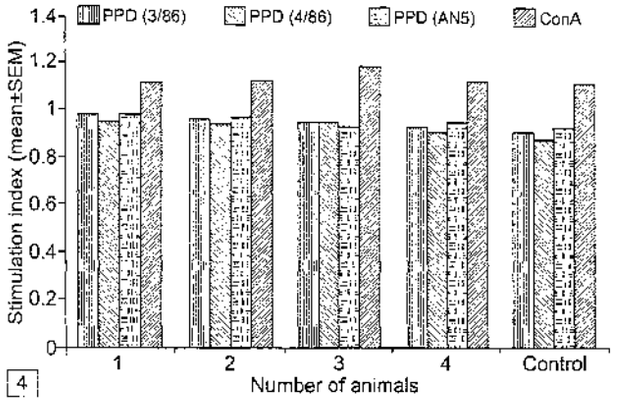
cattle calves. It was found that DTH skin responses against PPD *M. bovis* (3/86 and 4/86) was quite impressive in experimentally infected cattle calves and DTH responses with PPD *M. bovis* (3/86 and 4/86) were significant at 72 h post-inoculation of tuberculin (Figs 2–3).

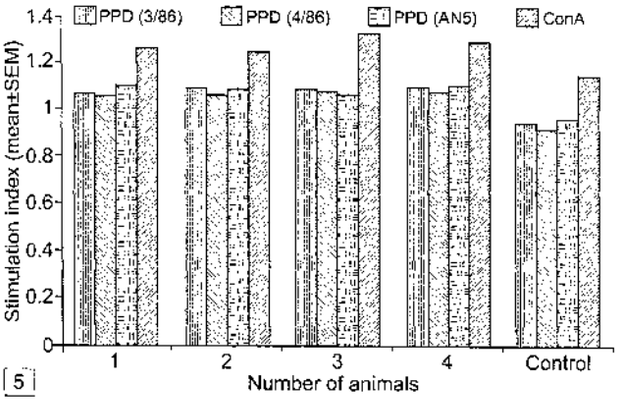
In vitro lymphocyte transformation test (LTT) was used to detect minute differences between closely related antigens (Rosenberg *et al.* 1972). LTT was used to compare the biologic activity of tuberculins (Nilsson and Magnusson 1973, Alhaji *et al.* 1974). In the present study, there was no significant variation of proliferative responses in PPD (*M. bovis* 3/86, 4/86) and PPD *M. bovis* AN5. Further, it was found that response to different PPDs increased from day 0 to day 42nd and the response was highest at day 42nd post-sensitized calves (Figs 4–7). The lymphoproliferative response with Con A was higher than PPD. It is reported that CD^{4+} T cells and γδ TCR cells are the predominate subsets of lymphocytes responding to PPD (Waters *et al.* 2000). We

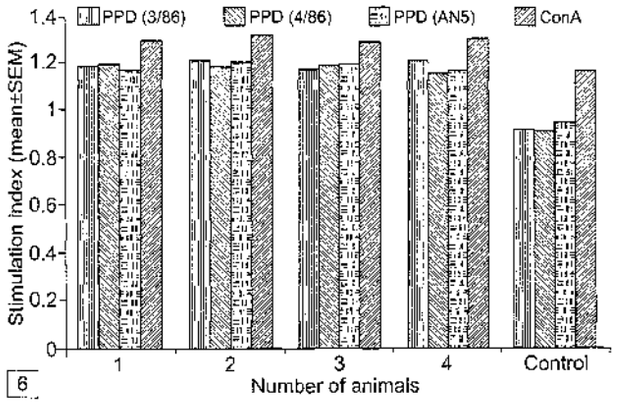




Figs 2–3. Comparative testing of experimentally infected cattle calves by SIDT using different PPDs: **2.** at the day of sensitization; **3.** after 45 days of sensitization.







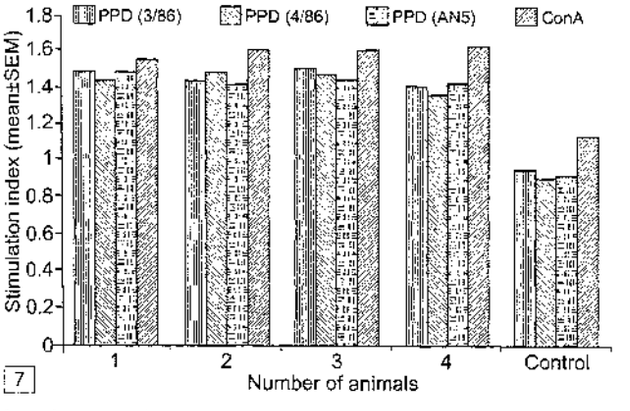


Fig. 4–7. Assessment of lymphocyte stimulatory activity by LTT (MTT dye reduction assay): **4.** Cell-mediated immuno-reactivity of PPD: at 0 day; **5.** at 14th day; **6.** at 28th day; **7.** at 42th day.

have not specifically dealt lymphoproliferative response of T or B cells, but it appears that the lymphoproliferative response to our PPD may be due to T cells. It is also reported that PPD, which is comprised mainly of denatured small molecular weight protein fragments, is a poor stimulant of B cell proliferation (Waters *et al.* 2000).

DTH and lymphocyte stimulation (*in vitro*) depend on the presence of sensitized lymphocytes in the circulation. A high degree of correlation is therefore expected between these two tests. There is, however, conflicting evidence on this point. Concerning tuberculin, which is a classic antigen in DTH, some authors have been able to demonstrate good correlation between *in vitro* tests and DTH (Hinz *et al.* 1970) whereas others have found no such correlation (Thomas *et al.* 1971). These discrepancies may be due to variations in experimental technique, e.g. PPD concentrations used and timing of the measurements of lymphocyte reactivity. A clear correlation was found between the result of DTH and the lymphocyte response *in vitro* against different PPDs, in this study.

The lymphoproliferative responses of PPD *M. bovis* 3/86 and 4/86, and AN5 were almost same, and response of Con A was statistically significant than all other PPD tuberculin's. It is reported that diagnostic tuberculin's are a complex, ill-defined mixture of both secreted and somatic mycobacterial antigens (Monaghan *et al.* 1994), which undergoes progressive proteolytic degradation. Our results share the view of Minden *et al.* (1986) that degradation does not jeopardize by itself the diagnostic efficiency of tuberculin, since peptides can be also very active in revealing DTH to mycobacterial antigens. Renato *et al.* (2007) reported that in Brazil indigenous production of PPD from the strains of *Mycobacterium tuberculosis* isolated from patients showed better results than PPD, which was distributed and imported by health system in all Brazilian regions. This supports our study that PPD produced from *M. bovis* 3/86, may be a potential candidate for the large-scale production of PPD, and tests performed for standardization of PPD in this study may serve as reference to the PPD production laboratories.

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