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Performance of Different Solid and Liquid Culture Media for the Improvement of Tuberculin Production

Shereen, AM[1], Marwah, M Mohamed[1], Abear S Almagraby[2] and Abdel Rahman M[1]

[1]Bacterial Diagnostic Products Department, Veterinary Serum and Vaccine Research Institute, Agriculture Research Centre, Cairo, Egypt; [2]Central Laboratory for Evaluation Veterinary Biologics, Agriculture Research Center, Egypt
***Corresponding author:** ryym_110@yahoo.com.

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ABSTRACT

Tuberculosis (TB) is one of the most important zoonotic bacterial diseases. A huge economic loss which could be direct or indirect are associated with the disease. Currently, the primary methods used for detection of TB in humans and cattle include the measurement of a delayed type hypersensitivity to purified protein derivative (PPD). So, the need for preparation of purified PPD with adequate properties and increasing the final PPD yield with decreasing the time of tuberculin production has stimulated the interest in the development of its preparation. Our study was performed to compare between the standard and modified media for improving tuberculin production. Middle brook 7H10 agar medium was used as a modified basic medium for mycobacterial growth, followed by cultivation of mycobacteria on Middle brook 7H9 broth medium. For the production, strains were inoculated onto the culture medium (Dorest Henly synthetic medium). Other steps for tuberculin production was done according to standard Weighbridge protocol. The results demonstrated that the using of both Middle brook 7H10 agar and Middle brook 7H9 broth instead of *Lowenstein-Jensen* (LJ) and glycerin broth media which used in currently produced tuberculin, have better physical and chemical properties. In addition, reducing the time required for production by accelerating the time of microbial growth. Also, it was found that the tuberculin produced using modified media was slightly more potent or the same as currently tuberculin produced. So, both Middle brook 7H10 agar and Middle brook 7H9 broth media are recommended for production of tuberculin saving time and increasing potency of the product but more investigation was recommended for estimation types of protein present in both locally prepared and modified tuberculin.

Key words: *Mycobacterium tuberculosis;* PPD; tuberculin; Middle brook 7H9; LJ

INTRODUCTION

Bovine tuberculosis (BTB) is a chronic bacterial disease of animals and humans. In many countries BTB is a major disease among cattle but transmission to humans is considered a public health threat where consumption of contaminated unpasteurized milk and other dairy products can be a source of human infection. Moreover, the important risk factor for BTB in humans is the exposure to aerosols containing *Mycobacterium bovis* (Martin *et al.*, 2012 and OIE, 2018). *M. bovis*, the main causative agent of bovine tuberculosis, it is a member of *Mycobacterium tuberculosis* complex (Smith *et al.*, 2006). Tuberculin Purified Protein Derivative (PPD) is a crude product obtained by precipitation of proteins from heated *Mycobacterium tuberculosis* strains cultures. The PPD is a very complex mixture of many soluble protein antigens and their aggregated and degradation products. In 1810, Robert Koch found that the boiled, crude extract of tubercle bacilli in glycerin (referred to as'' Old tuberculin'') can be used as a potential vaccine against tuberculosis infection. However, the old tuberculin of Koch's could not be used in vaccination due to its toxicity and impurity (Burke, 1993 and Shingadia and Novelli, 2008). The Tuberculin Skin Test (TST) has been used primarily as a standard method for diagnosis of tuberculosis by Von Pirquet in 1909 (Lee and Holzman, 2002). TST is an in vivo diagnostic test for detecting sensitization to mycobacterial antigens, which may result from infection, vaccination or environmental exposure (Mei *et al.*, 2006). The quality control testing of both PPD identity and potency depended on vivo assays which measure the ability of tuberculin to elicit delayed hypersensitivity reactions consequence to intradermal injection of guinea pigs which previously sensitized to mycobacterial antigens. The tuberculin skin reaction, based on the size of the zone of erythema and oedema at the

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injection site in comparison with a reference preparation, determines the identity and potency of the PPD preparations (WHO, 1987). The tuberculin skin test is important in the tuberculosis prevention programs. So, the need for the preparation of a more purified tuberculin with adequate properties and increasing the final PPD yield with decreasing the time required tuberculin production has stimulated the interest in its preparation. The purpose for conducting this study was to compare between the use of standard and modified media for improving the tuberculin production.

MATERIALS AND METHODS

Seed strains

Standard mycobacterial strains were used for production of tuberculin obtained from Central Veterinary Laboratory (CVL), Weighbridge, England.

Culture media used for production

a) Solid media

Lowenstein – Jensen medium (LJ) was prepared according to Collee *et al*. (1996).

Middle brook 7H10 agar medium enriched with oleic acid, albumin, dextrose, and catalase (OADC) growth supplement (Becton, Dickinson and Company, USA).

b) Liquid media

Seeding medium (glycerin broth medium) prepared according to the protocol of tuberculin production in CVL, Weighbridge, England.

Middle brook 7H9 broth medium enriched with oleic acid, albumin, dextrose, and catalase (OADC) growth supplement (Becton, Dickinson and Company, USA).

Synthetic medium (Dorset Henly Synthetic Medium).

Tuberculin purification (Weighbridge, 1970)

Cultivation and killing of Mycobacteria by autoclaving:

The LJ and Middle brook 7H10 slants were inoculated with standard strains at the same time and incubated at 37°C for 6-8 weeks. After that *M. tuberculosis* strains were propagated on glycerin broth and Middle brook 7H9 broth media and incubated at 37°C till the pellicle is fully grown on the media surface. This pellicle was transferred to Dorest Henly synthetic medium for 8 – 10 weeks. The inoculated flasks were observed daily during the first week and then once weekly for any contamination. Any culture flasks with submerged pellicle or showing abnormal growth or signs of contamination should be discarded. After the completion of cultivation, the cultures were autoclaved at 121°C for 30 min for killing of mycobacteria.

The other steps were taken as the follow:

Coarse Filtration of sterilized culture, proteins were precipitated using trichloroacetic acid (Green, 1953), proteins were purified according to CVL, Weighbridge, England, (1970) till having the PPD, PPD was dried in desiccator for 2months to remove all the humidity and the final product was dissolved in sterile phosphate buffered solution.

Quality control

The quality control was done according to OIE, 2015.

Sterility tests (OIE, 2015)

Was done by cultivation on different media (nutrient agar, glycerin broth, sabouraud's agar and thioglycollate media).

Protein content (OIE, 2015)

The protein content of the prepared tuberculin was estimated by using Semi – micro Kjeldahl method. Protein should not be less than 2mg / ml for tuberculin solution.

Physical stability (OIE, 2015)

The both prepared tuberculin were kept at 4C° (Nasr *et al.,* 2010).

Potency test (Dabbler *et al*., 1983)

Six sensitized albino guinea pigs to human mycobacteria are injected intradermaly with three dilution of the tuberculin produced by the routine method on one side in comparison with the same dilutions of tuberculin produced using the modified media on the other side. 24 hours post inoculation, the diameter of injected area is measured longitudinally and transversely, then potency of each tuberculin was determined by calculating the means of the diameters for each tuberculin concentration for all guinea pigs then take mean of all concentrations then divide the mean of the modified tuberculin and the routine tuberculin by the mean of standard tuberculin then multiply by 100, it will result in the potency.

RESULTS

It is cleared from Table (1) that the modified tuberculin was colorless while the locally prepared tuberculin have straw yellow color. The PH of both tuberculin were 7.2 and 7.0, subsequently. While, the both types of tuberculin were potent, stable and sterile. In addition, the obtained results in Table (2) showed that the both tuberculin have potent and nearly equal protein contents. Also, the result of biological potency using six guinea pigs as shown in Table (3), the potency of the modified tuberculin was equal to 111.5%. While, for the locally prepared tuberculin, the estimated potency was 106.4%.

DISCUSSION

Tuberculin was first prepared by Robert Koch in 1890 and was described as being a therapy for tuberculosis. His findings were the catalyst for the development of the modern TST, the most important tool for identifying potential TB cases up to date (Shingadia and Novelli, 2008). Shortly, there after the capabilities of PPD as a diagnostic material in animals were recognized. In 1934 Seibert and Glenn was prepared the first batch of a much more purified preparation, which they termed purified protein derivative (PPD). The tuberculin skin test, which involves monitoring the immune reaction to an injection of PPD, has been the most widely used method for detecting infection with *Mycobacterium tuberculosis* since its development in 1930s (Yang *et al*, 2012). For more than half a century the diagnosis of bovine tuberculosis depended

Table 1: Comparison between the routine and modified tuberculin depending upon physical and chemical properties.

Item	modified tuberculin	routine tuberculin
Appearance	Colorless	Straw yellow
PH	7.0	7.2
Potency	Potent	Potent
Sterility	Sterile	Sterile
Stability at 4-8 C°	Stable	Stable

Table 2: The result of purity test and proteins content of Mammalian tuberculin obtained from both seeding and Middle brook 7H9 broth media.

Tuberculin	Glycerin broth at 37℃ for 24hrs incubation	Thioglycollate medium at 37℃ for one-week incubation	LJ medium at 37℃ for 8- 12 weeks incubation	Sabouraud's agar at 25℃ for 15 days	Protein content mg/ ml
Standard from three patches	-Ve	-Ve	-Ve	-Ve	2.0002
Modified	-Ve	-Ve	-Ve	-Ve	2.0005

Table 3: Potency tests of standard, locally prepared and modified tuberculin.

Guinea Pigs	Standard tuberculin (mm)			Locally prepared tuberculin (mm)			Modified tuberculin (mm)			Total
	1/200	1/400	1/800	1/200	1/400	1/800	1/200	1/400	1/800	
1	22	12	13	23	18	15	23	18	15	159
2	20	16	11	21	17	12	21	17	13	148
3	21	15	11	22	16	11	23	19	15	153
4	20	14	13	20	16	15	24	17	13	152
5	21	18	13	22	19	14	22	19	12	160
6	20	17	11	21	18	11	24	18	13	153
Total	124	92	72	129	104	78	137	108	81	
Mean	20.7	15.3	12	21.5	17.3	13	22.8	18	13.5	

up on delayed type hypersensitivity. The composition of PPD is highly complex and remain ill- defined but recently found that the most prevalent antigenic proteins in PPD are now known to be the bacterial heat shock proteins (or chaperones) (Prasad *et al.,* 2013). The Weighbridge technique was considered the simplest and cheapest method of preparation of PPD tuberculin. The technique of Weighbridge, devised in 1939 and already in routine use since 1943, was published in The Veterinary Journal in September 1946. It consists of growing fixed laboratory strains on LJ medium for 6- 8 weeks then in glycerin broth medium (seeding medium) for 17-20 days followed by cultivation on synthetic medium for eight to ten weeks (Green, 1951). In recent years more attention has been devoted to liquid and solid media that offer significantly shorter times for the growth of mycobacteria (Palange *et al.,* 2016). The objective of this study depended upon use of different media than that used in the currently locally prepared PPD. The Middle brook 7H10 agar and middle brook 7H9 broth media were used at the same time with standard media (LJ and glycerin broth media) for cultivation of standard strains of *Mycobacterium tuberculosis* as the first step for tuberculin production. As shown in Table (1) the modified tuberculin was colorless while the locally prepared tuberculin have straw yellow color. The PH of both tuberculin were 7.2 and 7.0, subsequently. Generally, the physical properties of modified tuberculin is better than that of the local one.

The results of this study demonstrated that the time required for growth of standard strains on the modified media (Middle brook 7H10 agar and middle brook 7H9 broth media) is shorter than those required for growth using the Standard media (LJ and glycerin broth media). With subsequent decreasing the time required for tuberculin production. The obtained results are in agreement with results reported by Soto *et al.* (2009) who reported that the time required for the growth of *Mycobacterium tuberculosis* on middle brook 7H9 broth medium was significantly lower than that required for growth on Ogawa medium.

Previously it has been the custom to compare the qualities of purified tuberculin proteins prepared by different investigators, regardless of the fact that the components of a culture filtrate of tubercle bacilli varied considerably in quality and quantity according to the strain used, type of media and culture conditions (Toda *et al.,* 1959).

It was also shown from Table (2), that the both tuberculin have potent and nearly equal protein contents. Ideally, Tuberculin PPD potency should be evaluated in the species in which the tuberculin will be used but due to practical difficulties in performing potency assays in cattle, for routine PPD production, they are usually assayed in guinea pigs Duignan *et al* (2019). The result of biological potency using six guinea pigs as shown in Table (3), resulted in that the potency of the modified tuberculin was equal to 111.5%, this is within range estimated by CVL, Weighbridge that was between 80 and 120%and also agree with results of Ciuca *et al* (2015) who estimated potency between 66 and 150%. For the locally prepared tuberculin, the estimated potency was 106.4%. From the results it could be cleared that the modified tuberculin is more potent than routine tuberculin but both are potent. So this modified media could be recommended for tuberculin production saving time and more potent.

Conclusions

Form the results obtained in this study, it was observed that the using of Middle brook 7H10 agar and Middle brook 7H9 broth media in tuberculin production were recommended saving time and increasing potency of the product but more investigation was recommended for estimation types of protein present in both locally prepared and modified tuberculin.

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