

Study on the Physico-chemical Factors Augmenting the Growth of *Mycoplasma mycoides* subspecies capri

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Abstract

Different physical factors like aeration, temperature and shaking culture conditions were applied to the culture and observation of growth was studied for 72 hours of incubation. Similarly the *Mycoplasma mycoides* subspecies capri was grown in PPLO medium enriched with different concentrations (5, 10 and 20%) of serum from horse, bovine and allantoic fluid, along with usual concentration of yeast extract (1%) to record their effect on the growth. It was observed that growth was optimum (2.7×10^4 /ml) at 37°C in stable cultures under microaerophilic condition after 48 hours of incubation. The horse serum (20%) was suggested as an excellent biological material for enhancing the growth and replication of *Mycoplasma mycoides* subspecies capri (PG3). A direct positive correlation ($r^2=0.94$) was found between viable count and different protein concentrations of washed suspension of *Mycoplasma mycoides* subspecies capri.

Keywords: *Mycoplasma mycoides*, factors, growth

Introduction

Goat rearing carries tremendous importance in the rural economy particularly for non-agricultural poor people. Goat is recognized as poor man's cow in Pakistan. It provides milk, meat, skin, mohair and manure. The existing population of goats in Pakistan is 47.4 million and it provides about 28% of the total available meat (Anonymous, 2000).

Goat farming in Pakistan is confronted with the prevalence of many diseases among which caprine mycoplasmosis is of particular importance. This respiratory disease, owing to its high morbidity, lingering mortality and poor response to therapeutic agents remained consternation to the farmers and poses a significant threat for the rapid development of goat industry in Pakistan (Tariq, 1980).

The disease is global in its distribution. Amongst many species of mycoplasma, *Mycoplasma capri* causes contagious caprine pleuropneumonia (CCPP).

Little work has been done on the isolation and growth requirements of local isolates, probably due to their immense fastidiousness and superinfection by other bacteria that obscure the existence of mycoplasmas in the morbid tissues. The present study reports the physico-chemical factors which may enhance the growth of *Mycoplasma mycoides* subspecies capri (PG3) under *in-vitro* conditions. The results of the present study paved a way in the development of mass scale production of *Mycoplasma capri* for vaccine improvement.

Materials and Methods

Source of organism

Vaccinal strain of *Mycoplasma mycoides* subspecies capri (PG-3 strain) was procured from Veterinary Research Institute, Quetta, Pakistan and the culture was stored in pleuropneumonia like organism (PPLo) broth tubes at 4°C till required.

Cultivation and identification of pure culture

Mycoplasma broth base (Oxoid, UK) was reconstituted by dissolving 5.10 gm of base in 160 ml of distilled water. Two ml of thallium acetate (5%) along with 10 ml of horse serum was dissolved in 28 ml of separate distilled water. Broth base was autoclaved at 115°C for 15 minutes while thallium acetate and horse serum were filtered in Millipore membrane filter assembly through 0.22 µm APD nitrocellulose membrane. Penicillin-G (20,000 I.U) and streptomycin (20 mg) were added into the medium after filtration. Final medium was distributed into 20 sterilized screw capped test tubes and stored at refrigeration temperature for further use.

Activation of culture

One ml of broth culture of *Mycoplasma mycoides* subspecies capri was transferred aseptically into two test tubes containing fresh PPLo broth and incubated at 37°C for 10 days. Growth was observed daily in terms of whorl formation and turbidity.

Identification of culture

Growth from broth inoculated tubes was transformed onto the brain heart infusion Agar (BHIA), blood agar (BA), PPLo agar with phenol red and PPLo agar with tetrazolium dye. After incubation at 37°C for 48 hours the growth with typical colony characteristic was

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observed on each medium. Morphological examination of typical mycoplasma growth from the surface colony was observed by Geimsa staining method (Buxton and Fraser, 1977).

Confirmation of culture

Culture obtained from typical colony on surface of the PPLO agar were transferred to fresh PPLO broth in duplicate test tubes and specific growth was reconfirmed through morphological examination after 72 hours of incubation at 37°C. The pure growth was processed for biochemical and sugar fermentation tests as described by Al-Aubaidi *et al.* (1972). Serological identification was also conducted using specific known antiserum obtained from the Department of Veterinary Microbiology, University of Agriculture, Faisalabad, Pakistan. The antiserum was raised in rabbits against the known antigen of *Mycoplasma mycoides* subsp. *capri* strain PG3.

Physico-chemical factors

PPLO broth base medium prepared as described earlier was inoculated with 1 ml of pure *Mycoplasma capri* (PG3) in triplicate set of test tubes placed under 30°C and 37°C separately to observe the temperature of incubation upto 96 hours. Broth was observed daily in terms of turbidity, colour change and whorl formation. Moreover, viable number of bacteria were also recorded according to the multiple dilution method on PPLO agar plate containing tetrazolium dye as described by Awan and Rahman (2002).

An other set of triplicate test tubes containing PPLO broth were inoculated with 1 ml of activated growth of *Mycoplasma capri* (PG3) and placed under two different set of conditions including shaking culture condition at (80 rph) as well as under stable culture condition. Both set of test tubes were incubated at 37°C for 96 hours.

Selecting the best temperature conditions along with physical culture conditions were provided to the growth of *Mycoplasma capri* (PG3). Three set of duplicate test tubes containing PPLO broth were maintained with different source of serum enrichment including horse serum, bovine serum and egg albumin with each having 5, 10 and 20% (v/v) concentration separately. After inoculation the results were recorded in terms of turbidity, whorl formation and viable bacterial count at the end of the study.

Standardization of *Mycoplasma capri* growth at various protein concentrations

Mycoplasma capri (PG3) was grown into 4 liter of PPLO broth medium enriched with 20% of horse serum and incubated under stable condition for 48 hours at 37°C. The growth was confirmed by cultural and morphological examination. Whole culture suspension was pelleted at 22,000 Xg. Homogeneous suspension of *Mycoplasma capri* was prepared in normal saline

solution upto 100 ml volume. Protein concentration was determined by the method described by Bradford (1976) and viable number of bacteria from the fresh suspension were also studied using the method of Awan and Rahman (2002) in the respective dilution of 1, 2, 3, 4 and 5 gm/100ml of total protein. Standard curve was plotted between protein concentrations and viable count and correlation coefficient was calculated.

Results and Discussion

It was recorded that temperature of 37°C showed whorl formation after 24 hours of incubation without standing turbidity, while the incubation temperature of 30°C showed whorl formation without standing turbidity after 48 hours, while the turbidity started after 72 hours of incubation and there was no change in colour of the medium even after 96 hours of incubation. Moreover, the incubation temperature of 37°C over the time of 24 hours showed the appearance of whorl formation along with the turbidity. There was change in colour of the broth to slight pinkish after 96 hours of incubation (Table 1).

Table 1: Effect of temperature on the growth of *Mycoplasma mycoides* subspecies *capri* in *Mycoplasma* broth base

Incubation temperature	Incubation time (hour)	Whorl formation	Turbidity	Colour change
30°C	24	---	Nil	No
	48	+	Nil	No
	72	++	Positive	No
	96	++	Positive	Yellow
			Viable count: 2.3×10^4 /ml	
37°C	24	+	Nil	No
	48	++	Positive	No
	72	++	Positive	Slight Yellow
	96	++	Positive	Slight Yellow
			Viable count: 2.7×10^4 /ml	

The optimum growth of *Mycoplasma mycoides* subspecies *capri* was obtained at 37°C in contrast to 30°C. Similar temperature range of 36-38°C have been suggested by Robinson and Tully (1998) for the optimum growth of *Mycoplasma* and *Ureaplasmas* of human and animal origin. Enhancement of growth at 37°C was further evident from the highest viable count of *Mycoplasma mycoides* subspecies *capri* (2.7×10^4 /ml) in contrast to 30°C of incubation for 48 hours.

Table 2: Effect of continuous shaking culture on the growth of *Mycoplasma mycoides* subspecies *capri* (PG3) at 37°C temperature in Mycoplasma both base

Parameters	Time of incubation under various conditions (hours)							
	24		48		72		96	
	Shake	Stable	Shake	Stable	Shake	Stable	Shake	Stable
Whorl formation	-	+	-	+	+	+	-	+
Turbidity	-	+	-	++	++	++	++	++
Colour change	-	-	-	-	Slight change		Slight change	Yellow colour
Viable count (ml)	<10 ¹	<10 ¹	<10 ¹	<10 ¹	1.7×10 ²	2.1×10 ³	1.5×10 ³	2.7×10 ⁴

Table 3: Effect of different concentration of bovine, equine serum and egg albumin in Mycoplasma broth base on the growth of *Mycoplasma mycoides* subspecies *capri* (PG3) incubated at 37°C

Source of serum	Concentration used in PPLO broth (%age)	Incubation period	
		24 hours (Whorl/Turbid)	48 hours (Whorl/Turbid)
Horse serum	5	Nil/Nil	Nil/Nil
	10	-IV/Nil	+IV/-IV
	20	+IV/-VE	++IVE/++IVE
	Viable count: 1.1 × 10 ⁶ /ml		
Bovine serum	5	Nil/Nil	Nil/Nil
	10	Nil/Nil	+IV/Nil
	20	+IVE/-IVE	++IVE/++IVE
	Viable count: 2.3 × 10 ⁵ /ml		
Egg albumin	5	Nil/Nil	Nil/Nil
	10	Nil/Nil	+IVE/-IVE
	20	+IVE/+IVE	++IVE/+IVE
	Viable count: 1.3 × 10 ⁴ /ml		

The stable culture showed maximum recovery of viable bacteria in contrast to continuous shaking condition which might be microaerophilic that have supported the better multiplication of *Mycoplasma* in the medium. According to McGarrity et al., (1983) the most of *Mycoplasma* species and several unclassified strains require reduced environment for both primary isolation and maintenance.

Mollicutes are deficient in their ability to synthesize some of the important components of their membrane like saturated, unsaturated fatty acids and cholesterol, therefore, the addition of 10-20% of horse serum is suggested by McElhaney (1992). Maximum viable count of *Mycoplasma mycoides* subspecies *capri* (1.1×10⁶/ml) was found after 48 hours of incubation in PPLO broth containing 20% horse serum in contrast to bovine serum and egg albumen (Table 3). From the results horse serum (20%) suggested as an excellent biological material for enhancing the growth and replication of *Mycoplasma mycoides* subspecies *capri* (PG3). However, Tariq (1980) suggested the use of bovine serum for the growth of *Mycoplasma capri*.

The total protein contents of washed suspension of *Mycoplasma mycoides* subspecies *capri* were 5.2g/100 ml and viable count was 2.4x10¹⁸/ml. Washed suspension diluted in normal saline solution maintained

the protein concentration at 1,2,3,4 and 5 g/100ml. Each of diluted suspension was found to have a direct positive correlation with the viable count with a correlation coefficient (r²=0.94) at P<0.01%.

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