MOLECULAR CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* ISOLATED FROM AN INCIDENCE OF SHEEP PASTEURELLOSIS IN KARAMADAI HILL TRACT OF TAMIL NADU

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ABSTRACT

Sheep Pasteurellosis was recorded in temperate, subtropical areas of karamadai hill tracts of Coimbatore district in Tamil Nadu. Seventy two nasal swab samples from sheep origin were collected and six isolates of P.multocida *were obtained which evinced characteristic morphological and cultural properties. Necropsy findings revealed typical haemorrhagic lesions and bipolar organisms, suggestive of pasteurellousis.* Pasteurella multocida *was isolated from heart blood and tissues and characterized. PM-PCR using the specific primers confirmed the isolates as* P.multocida *and by Capsular PCR all isolates were serotyped as capsular type 'A'.*

Key Words: Sheep pasteurellosis, *Pasteurella multocida,* PM-PCR

INTRODUCTION

Pasteurellosis caused by *P.multocida* is an acute septicaemic disease characterized by high morbidity and mortality in cattle, sheep, goat and poultry resulting in economic losses. Sheep pasteurellosis is one of the common infectious and economically important bacterial diseases which occur in temperate, subtropical areas (Chandrasekaran et.al.,1991) and affects all age group of sheep leading to unthriftiness reduction in body weight and death. Pasteurellosis in sheep is serotype specific and it is necessary to monitor continuously the prevalence and emergence of various serotypes as this overall assessment can lead to production of an efficient vaccine. This study is based on an occurrence of virgin outbreak of sheep pasteurellosis in the karamadai hill tract of Mettupalayam region in Coimbatore district, Tamil Nadu. It was reported that few deaths of sheep and lambs with pneumonic symptoms of suspected pasteurellosis in February 2007 were recorded by NGO based in Krishi Vigyan Kendra, Avinasilingam college, Coimbatore.

MATERIALS AND METHODS

The authors visited the outbreak areas of Karamadai hill tracts and collected specimens from suspected cases of ailing, apparently healthy and dead animal. Seventy two nasal swab samples were collected from ailing sheep at eight hamlets in Karamadai hill tracts of Coimbatore district in Tamil Nadu. The ailing sheep exhibited pneumonic symptoms, respiratory distress, profuse nasal

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discharge and sneezing. Heart blood and tissue pieces of spleen, liver and lungs were collected from dead animals.

Mouse bioassay:

The specimens were segregated and pooled into eight different groups and inoculated in BHI broth. After 16hrs incubation at 37°C each grouped inoculum(0.1ml) was injected into a set of Swiss Albino Mice. The Heart blood smears and impression smears of spleen, liver and lung were collected from dead mice.

Aspirated heart blood from dead mice was streaked onto 10% sheep blood agar and incubated at 37°C. The heart blood was inoculated in BHI broth also and incubated at 37°C for 16hrs and the broth culture was streaked onto blood agar and MacConkey agar

Biochemical tests

Biochemical tests were carried out using Entero rapid – 24 test kit as described by Tefera (2002).

Antibiotic sensitive assay:

Antibiotic sensitive assay was performed for the *P.multocida* isolates with 14 antibiotics as per the disc diffusion method of Kirby-Bauer(Bauer *et.al.,*1966).

DNA extraction by high salt method:

DNA was extracted from the culture by high salt method as described by Fischer and Lerman(1979). Overnight culture was centrifuged at 10,000 rpm for 20 min. The pellet was washed with PBS and twice with 0.5 ml of solution I(10mMTris Hcl, 10mMKcl, 10mM $Mgcl_2$,2mM EDTA). The final pellet was resuspended in 0.5ml of solution II(10mMTris Hcl, 10mMKcl, 10mM $Mgcl_2$,2Mm EDTA,0.4M NaCl) and incubated at 37⁰C for 15 min in water bath. 50µl of 10% SDS and 250µl of 6 M NaCl was added and centrifuged at 10,000 rpm for 5 min at 4⁰C and then precipitated with ethanol. The resulting pellet which contains DNA was resuspended in Low Tris EDTA (LTE) buffer and stored at –20⁰C until used.

Culture Lysate Method:

Two ml of 18hrs broth culture was pelleted by centrifugation at 10,000 rpm for 10 minutes. The pellet was washed with sterile PBS twice and resuspended in 30µl sterile nuclease free water and boiled for 10 minutes. After boiling, the sample was snap chilled over ice. The samples were centrifuged at 3000rpm for 10 minutes to sediment the cell debris and supernatant stored at –20⁰C until used.

***P.multocida* species specific PCR (PM-PCR)**

The species specific primers designed by Townsend *et.al.,* (1998a) KMTIT7 and KMTISP6 were used to amplify the gene sequences in *P.multocida,* The PCR reaction mixture and the thermal cycle protocol were as follows Initial denaturation – 94⁰C for 5 min, followed by 30 cycles, each cycle consisting of 3 steps denaturation at 95⁰C for 1 min, annealing at 55⁰C for 1 min, Extension at 72⁰C for 1 min. Final Extension at 72⁰C for 9 min.

Capsular PCR typing

The *P.multocida* capsular serogroup specific primers designed by Townsend *et al.* (2001) were used for capsular PCR typing. The serogroup A specific primers *hya* D and *hya* C were used to amplify capsule biosynthetic loci of serogroup "A". The thermal cycle protocol were as follows Initial denaturation at 95°C for 5 min, followed by 30 cycles, each cycle consisting of 3 steps denaturation at 95⁰C for 30 sec, annealing at 49⁰C for 30 sec, Extension at 72⁰C for 80 sec. Final Extension at 72⁰C for 5 min.

RESULTS AND DISCUSSION

Pasteurellosis caused by *P.multocida* is an opportunistic respiratory pathogen of sheep in tropical climate causing high morbidity and mortality. The predisposing factors include the hot tropical climate and stress induced by management practices such as docking, drenching, castration etc, as reported by Chandrasekaran *et.al.,* (1991)
A total of 72 samples suspected for pasteurellosis were pooled area wise into 8 groups and were subjected to biological test in mice and 6 isolates of *P.multocida* were obtained.

Mouse bioassay:

Mouse bio assay revealed that five *P.multocida* isolates were found to be virulent with Mean Death Time (MDT) between 12-18 hrs. One isolate had a MDT between 19 and 24 hrs. The mouse bio assay findings were comparable with the findings of Mustafa *et al* (1978), Diallo *et al* (1995) and Suresh babu (2003).

Heart blood smear, Liver and spleen impression smear evinced characteristic bipolar organism on Leishman staining. The six isolates showed typical morphological and cultural characteristics of dew drop, mucoid, non haemolytic colonies in blood agar. No growth was observed in MacConkey agar. Culture smears revealed characteristic Gram negative coccobacillary organisms These observation were in accordance with observation of Holmes (1998) and OIE manual (2004).

Biochemical tests

The isolates subjected to biochemical tests were positive for Indole, Nitrate reduction, Oxidase and Catalase and negative for MR test, VP test and Simmons citrate test. The isolates fermented glucose, fructose, Mannitol, trehalose and sucrose(Table-1). These results are in accordance with Tefera(2002) and OIE manual (2004).

Antibiotic sensitive assay:

In clinical aspects antibiotic sensitive assay serves as a guide to choose the correct antibiotic to be used in the field.(Coates and Hoops 1980).Antibiotic assay revealed 100% Sensitivity to ciprofloxacin, 83% sensitivity to ofloxacin, enrofloxacin and gentamicin. 67% sensitivity to azithromycin, norfloxacin, tetracycline, streptomycin in the order of preference. Hundred percent resistance was observed to amoxyclav and sulphadiazine(Table-2). Antibiotic sensitive results of this study are in accordance with Kawamoto *et.al.,* (1990).

DNA Isolation:

The isolates subjected to DNA isolation by high salt method and culture lysate method yielded the same result which was visualized by PCR. The amplicon size was found to be 460bp for both the template DNA obtained by high salt method and culture lysate method. Therefore DNA extraction by culture lysate method has been found to be simple and less time consuming when compared to high salt method.

P.multocida species specific PCR (PM-PCR):

Pasteurella multocida species specific PCR(PM–PCR) assay developed by Townsend *et.al*(1998) was used for this study to identify the subspecies of *P.multocida* by amplifying 460 bp DNA fragment within KMTI gene using the Primers KMTISP6 and KMTIT7.Based on standard molecular weight marker(100bpMarker),the molecular weight of the PCR products of all the isolates and reference strain (p52) was found to be 460 bp, specific for *P.multocida* in correlation with the findings of Townsend *et al* (1998) (Fig–1).

Capsular PCR typing

Capsular PCR method was designed to amplify the hyaC – hyaD gene of *P. multocida*. This gene was an ideal amplification target for PCR because hyaluronic acid is a principal component of type A

Table -1
Biochemical characteristics of *P.multocida* isolates

Samples	Indole	MR test	VP test	Simmons Citrate	H_2S Production	Nitrate Reduction	Lysine decarboxylase	Catalase	Ornithine decarboxylase
Sheep – 1	+	-	-	-	+	+	-	+	+
Sheep – 2	+	-	-	-	+	+	-	+	+
Sheep – 3	+	-	-	-	-	+	-	+	+
Sheep – 4	+	-	-	-	-	+	-	+	+
Sheep – 5	+	-	-	-	-	+	-	+	+
Sheep – 6	+	-	-	-	+	+	-	+	+

Samples	Oxidase	Urease	ONPG	Phenylalanine	Esculin hydolysis	Arginine	Fructose	Mannitol	Trehalose	Dulcitol	Glucose
Sheep – 1	+	-	+	-	-	-	+	+	+	+	+
Sheep – 2	+	-	+	-	-	-	+	+	+	-	+
Sheep – 3	+	-	+	-	-	-	+	+	+	-	+
Sheep – 4	+	-	+	-	-	-	+	+	+	+	+
Sheep – 5	+	-	+	-	-	-	+	+	+	+	+
Sheep – 6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Table – 2
Susceptibility pattern of *Pasteurella multocida* isolates to various chemotherapeutic agents

S.No.	Antibiotics	Concentration /disc	No. of isolates Resistance	No. of isolates Sensitive	Percentage of Sensitivity
1.	Amoxyclav	10 mcg	6	0	0
2.	Azithromycin	15 mcg	2	4	67
3.	Cephalothin	30 mcg	2	4	67
4.	Co-trimoxazole	25 mcg	3	3	50
5.	Ciprofloxacin	30 mcg	0	6	100
6.	Enrofloxacin	10 mcg	1	5	83
7.	Gentamicin	10 mcg	1	5	83
8.	Metronidazole	5 mcg	4	2	33
9.	Norfloxacin	30 mcg	2	4	67
10.	Ofloxacin	5 mcg	1	5	83
11.	Streptomycin	10 mcg	3	3	50
12.	Sulphadiazine	300 mcg	6	0	0
13.	Tetracycline	30 mcg	2	4	67
14.	Trimethoprim	5 mcg	4	2	33

Fig 1
Pasteurella multocida - Polymerase Chain Reaction (PM-PCR)

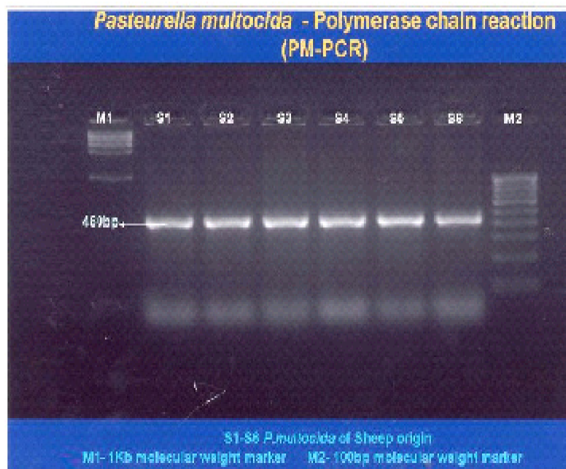
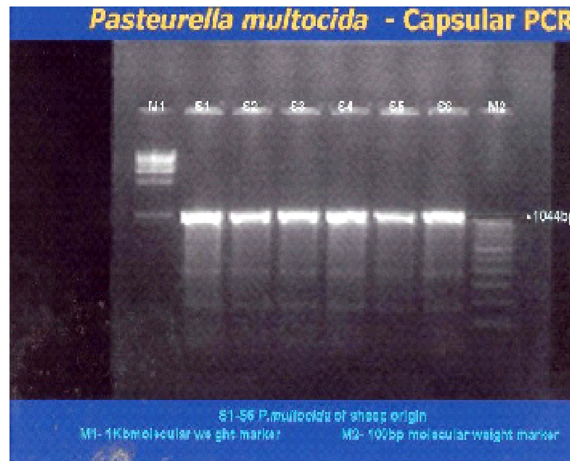


Fig. 2
Pasteurella multocida - Capsular PCR



capsule. All the six isolates used in this study all isolates were identified as capsular type 'A' The amplicon size was found to be 1044bp(Fig-2). Similar results had been observed by Jaglic *et al.* (2005) and Chung *et al.* (1998). All the six isolates were serotyped as capsular polysacc type A at Indian Veterinary Research Institute (IVRI), U.P.

CONCLUSION

Seventy two nasal swab samples were collected from sheep in karamadai hill tracts of Coimbatore district in Tamil Nadu. Six isolates of *P.multocida* were obtained. Cultural and biochemical characters revealed the presence of *P.multocida*. PM-PCR using the specific primers confirmed all the isolates as *P.multocida.* All the isolates were serotyped as capsular type 'A' by Capsular PCR and serotyping.

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REFERENCES

Bauer.A.W, Kirby.W.M.M *et.al,* (1966). Antibiotic susceptibility testing by a standardized single disc method. American Journal of Clinical Pathology., 45:493

Chandrasekaran.s *et.al*, 1991. Evaluation of combined Pasteurella vaccines in control of sheep pneumonia. British veterinary journal., 147:437-443.

Chung, Y.J. Zhang, Y. and Adler, B. 1998. The capsule biosynthetic locus of ***Pasteurella multocida*** A:1 FEMS ***Microbiology Letters*** 166 **:** 289-296.

Coates,S.R and Hooper 1980. Sensitivities of *E.coli* isolated from bovine and procrine enteric infection to antimicrobial antibiotics. American journal of veterinary research ,41:1882-1883.

Diallo et al (1995). Molecular studies on avian strains of *P.multocida* in Australia Veterinary Microbiology., 46:335-342.

Fischer, S.G and Lerman, L.S 1979 Length independent separation of DNA Restriction fragments in two dimensional gel electrophoresis cell,16:191.

Holmes, B. 1998. *Pasteurella In:* Topley and Wilson's microbiology and microbial infections. 9th Edn. Balow, A and B.I. Overden, Systematic bacteriology. 2nd Volume, Oxford University Press, New York

Jaglic, Z., Z.Kucerova, K. Nedbalcover, I. Pavlik, P. Alexa and M Barbs. 2005. Characterization and comparison of *Pasteurella multocida* isolated from different species in the Czech Republic : Capsular PCR typing, ribotyping and dermonecrotoxin production. Veterinary Medicine Czech. 50 :345-354.

Kawamoto,E. *et.al*,1990. Prevalence and characterization of *P.multocida* in rabbits and their environment in Japan. Japan Journal of Veterinary Science **.**,52:915-921.

Mustafa et al (1978) Carrier rate of *P.multocida* in a cattle herd associated with an outbreak of Haemorrhagic septicaemia in Sudan. British Veterinary Journal.,134:375- 380.

Office International des epizooties.2004. Manuals of standards for diagnostic test and vaccine. 4th edition, France, pp 446 – 456, 740 – 746, 921 – 926.

Prabhakar.T.G. 1995 Study on the toxigenic Strains of ***P.multocida*** Ph.D.**,** thesis submitted to TANUVAS.

Suresh babu, 2003. Studied on the carrier status of pasteurellosis in animals and birds M.V.Sc. thesis Submitted to TANUVAS.

Tefera.J.Smola(2002).Differentiation of *Pasteurella multocida* and *Pasteurella haemolytica* using Entero Rapid-24 Test kit. Veterinary Medicine Czech47(4)99-103

Townsend,K.M., Frost.A.J., Lee.C.W., Papadimitriou.J.M., Dawkins.H.J.S., (1998) Development of PCR assays for Species and type specific identificationof ***P.multocida*** isolates Journal of Clinical Microbiology, 36:1096-1100

16. Townsend, K.M., Boyce, J.D., Chung, J.Y., Frost, A.J. and Alder, B. 2001. Genetic organization of *Pasteurella multocida* cap loci and development of multiplex capsular PCR typing system. ***Journal Clinical Microbiology*** 39 **:** 924-929**.**