International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
DOI: 10.22192/ijarbs Coden: IJARQG(USA) Volume 3, Issue 9 - 2016

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2016.03.09.031

Molecular typing of Indian isolates of *Pasteurella multocida* serotype B:2 by ERIC- PCR

M.K. Saxena, A. Kumar, V.P.Singh, P. Chaudhari, S.B. Shivachandra and B. Sharma*

Division of Bacteriology and Mycology, Division of Biochemistry
Indian Veterinary Research Institute, Izatnagar – 243 122 (U.P.) India
* Corresponding author: **M.K.Saxena**
Animal Biotechnology Center, College of Veterinary and Animal Sciences
G.B.P.U.A&T-PANTNAGAR-263145, India
E-mail : *mumtesh@rediffmail.com* Fax: 0091-5944-233473 Telephone: 91-9458269455

Abstract

Hemorrhagic septicemia is an important disease of livestock in Asian continent. The present study was undertaken to assess the presence of *Pasteurella multocida* serotype B:2 in various species of animals and to assess the genetic variation among the field isolates of *Pasteurella multocida targeting* Enterobacterial intergenic consensus sequence (ERIC). Samples were collected from dead and live (suspected for HS) animals and organism was isolated and were molecular typedby ERIC-PCR. *Pasteurella multocida* serotype B:2 was isolated from cattle, buffalo, sheep, goat, pig and wild animals (loin and tiger). Isolates (42) were differentiated into 22 different profiles. Study indicate high genetic diversity, no geographic or host specificity among field isolates of *Pasteurella multocida* serotype B:2 . Vaccine strain P_{52} used for preparation of killed vaccine in India require re-evaluation since it was found to be distantly related to field isolates.

Keywords: *Pasteurella multocida*, ERIC-PCR, Hemorrhagic septicemia, repetitive elements, Vaccine.

1. Introduction

Pasteurella multocida is one of the most important gram negative bacteria affecting livestock in South East Asia. It causes disease in both domestic (bovine, porcine, ovine and poultry) and wild animals. It has 5 capsular and 16 somatic antigens (Carter, 1955). *Pasteurella multocida* is usually characterized serologically which has shown prevalence of specific serovars in different geographical regions (Gunawardana et al., 2000). In India haemorrhagic septicemia (an acute fatal septicaemic disease of cattle and buffalo) caused by *P. multocida* is usually attributed to serotype B: 2. A network project to control hemorrhagic septicemia was started in India.

Under this project large number samples were collected from reared and wild (dead) animals. The objectives were to assess the presence of serotype B:2 in different animals species and to analyze DNA variation among the isolates with reference to vaccine strain (P_{52}).

The DNA analysis is now increasingly being used in bacterial taxonomy and typing (Gulter and Myall, 2001, Klein et al., 1998). Polymerase chain reaction (PCR)-based techniques has been extensively used for detection and differentiation of pathogens (Shivshankar et al., 2001; Saxena et al., 2006;

Shivchandra et al., 2006 Saxena et al., 2004, Nagagapa et al., 2007). One of the methods for bacterial typing uses short interspersed repetitive elements present in many bacterial species. The REP (repetitive extragenic palindromic) and ERIC (Enterobacterial intergenic consensus sequence) are found in many copies dispersed around the chromosomes in the genomes of enterobacteria like *Escherichia coli* (Hilton et al.,1991) and *Salmonella* Typhimurium (Gilson et al.,1984). ERIC sequences which are interspersed repetitive (IR) DNA sequences were identified by Sharples and Loyd, 1990. This short repetitive sequence contains highly conserved central inverted repeats located in extragenic region and appears not to be related to other interspersed elements such as Repetitive Extragenic Palindromic (REP) sequence. Primers for ERIC-PCR have been designed to match the inverted repeat sequence and are directed outwardly (Verosalovic et al., 1991). Reason for designing such primers was the assumption that repetitive sequences are dispersed in different orientations and are separated by various distances. The primers would bind to them and give distinct product profiles. These primers have been used for strain differentiation in *Pasteurella multocida* serotype A:1 (Shivchandra et al.,2008**)** . Variation in the copy number of ERIC-sequence and inter-ERIC distance has been reported not only between the species but also within the species, serovars and serotype (Verosalovic et al., 1991). Present study was undertaken to assess genetic variation among different isolates isolated from different animals with special reference to vaccine strain $P_{52}$of India.

2. Materials and Methods

P.multocida isolates included in this study were isolated from domestic animals birds and wild animals. Nasal swab and blood were collected from clinically ill domestic animals. From dead animals (wild and reared) heart blood, spleen, liver and bone marrow were collected for isolation of causative agent. Each sample was inoculated in brain heart infusion medium (BHI broth, Difco)) at 37^0 C for 18 hours subsequently , broth culture was streaked on sheep blood agar and colony showing characteristic features of *Pasteurella multocida* were selected and preliminary screening was conducted on the basis of Gram's staining .

2.1 Screening of isolates by PM-PCR

Pasturella multocida specific PCR (PM-PCR) was performed on all the isolates (Townsend et al., 1998) briefly, single colony was inoculated in 2 ml of BHI broth and incubated at 37^0C for 18 hours. Culture was centrifuged at 3000g and the pellet was re-suspended in 100µl of sterilized water. Cell lysis was carried out by heating in boiling water bath for 10 minutes followed by immediate chilling on ice. The tubes were centrifuged at 12000g for 10 minutes. 10 µl of supernatant was used in 25µl reaction mixture containing 1XPCR buffer, 200µM of each dNTPs , 2U of Taq Polymerase , 20 pmol of each primers (Townsend *et al.* 1998). PCR was performed on PTC-200 (M J-RESEARCH) thermal cycler with an initial denaturation at 94^0C for 5 minutes followed by 30 cycles of denaturation at 94^0 C for one minute, annealing at 57^0C for 2 minutes and polymerization at 72^0C for 2 minutes with a final extension at 72^0C for 5 minutes. 5µl of amplified product was run in 1.5% agarose gel. Electrophoresis was conducted in IX TAE buffer at 5 volts/cm for two hours. Sizes of amplicons were compared with standard molecular weight marker 100bp ladder plus (MBI Fermentas).

2.2 Serotyping of *Pasteurella multocida*

PM-PCR positive were sent to Veterinary Research Institute Perideniya , Srilanka for serotyping.

2.3 ERIC-PCR

Genomic DNA was isolated from all the isolates by C-Tab method (Wilson 1987). ERIC-PCR was performed in 25µl reaction mixture containing 50 ng genomic DNA, 1µl (20 pmol) of each primer (Shivchandra et al., 2008), 2.5µl of 10X PCR buffer containing 1.5 mM $MgCl_2$ (MBI-Fermentas), 5µl (200µm) of each dNTPs (MBI-Fermentas) and 2.0U of Taq Polymerase (MBI-Fermentas). PCR was performed in a Thermalcycler (PTC-200MJ research) with an initial denaturation at 95^0C for 5 min and 30 cycles of denaturatrion (94^0C. 1min), annealing (51^0C, 2min) and extension (72^0C, 6 min) and a final extension step (72^0C, 10 min). ERIC-PCR was also conducted with bacterial cell lysate. Bacterial cell lysate was prepared by harvesting cells from 1.5 ml overnight grown culture in BHI broth. The cells were suspended in 500µl of triple distilled water. Suspension was boiled at 100^0C in water bath for 10 min followed by immediate chilling on ice. Cell debris was removed by centrifugation at 12000Xg for 5 minutes. 10µl supernatant was used as template DNA. Amplified products were electrophoresed in 1.5% agarose gel at 5 volt/cm for five hours in TAE buffer and molecular weight of amplicons were calculated by comparing with 100 bp (plus) ladder

(MBI Fermentas) . Dendrogram was constructed using band profile data of different isolates. To assess genetic variation among isolates with reference to vaccine strain similarity index was calculated (Lubinoux et al.,1998).

Results and Discussion

Pasteurella multocida Serotype B:2 was isolated in from states of India viz., Uttar Pradesh, Maharashtra, Andhra Pradesh, Punjab, Tamil Nadu and Delhi encompassing whole of India and most productive regions. HS is considered a disease of cattle and buffalo, however many isolates of serotype B: 2 were from pigs, sheep and goats, which indicate that host range of organism has become broader.

ERIC sequences were present in all the 42 isolates as amplicons were observed in all the isolates. Size of amplicons varied from 256 bp to 2990 bp (Fig-1) . There was not a single band which was present in all the isolates indicating existence of large genetic diversity among the isolates which was also reflected in the large number of profiles (22 profiles from 42 isolates) obtained after clustering (Fig-2). Similarity with the reference vaccine strain P_{52} with isolates from different species varied differently. The maximum variation was noted in isolates from cattle in which similarity with vaccine strain was from 15 to 83 %. The similarity with vaccine strain of isolates from other species were 38 to 100%, 52.6 to 85.7%, 58% and 64.8 to 90% for isolates from buffalo, pig, wild animals and sheep and goat respectively. ERIC-PCR has been shown earlier to be an efficient method for molecular typing of *Pasteurella multocida*. (Leottal et al.,2006, Shivchandra et al.,2008). We had obtained D value of 0.89 (Hunter and Gaston, 1988) obtained in this study also indicated high discriminatory power of ERIC-PCR.

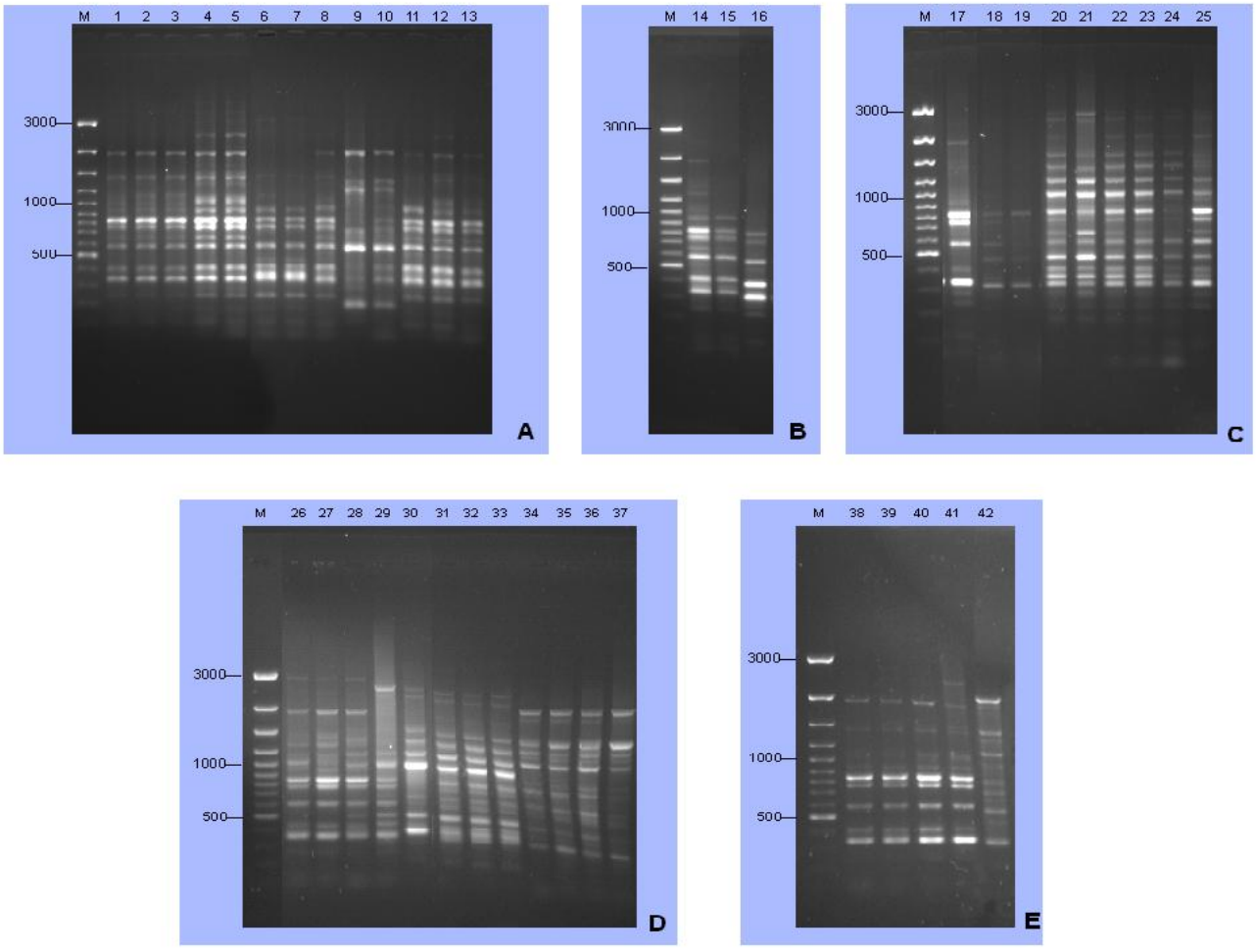

Fig.1.ERIC Finger Prints of Indian isolates of Pasteurella multocida serotype (B:2)
Lane M: Marker 100 pb (plus) (MBI Farmentas) Lane 1-42 : ERIC Finger prints of Indian islates of Pasteurella multocida serotype (B:2)

Geographical specificity of *Pasteurella multocida* has been reported (Gunwardana et al., 2000) but in present study we could not observe any profile which could be considered as specific for particular geographical area. There was also no host specific profile. Similar profiles were observed in isolates isolated from wild and reared animals. Presence of similar profiles in wild and reared animals(Table-1) indicate that organism is crossing species barrier and may get transfer to wild from reared animals and vice versa. Cluster analysis had shown that vaccine strain P_{52} was genetically similar with very few isolates (Fig-2). The vaccine strain p52 is a B:2 serotype of *Paseurella multocida* and is the only strain used in vaccine formulation in India. The results indicate that P_{52} vaccine strain is probably outdated now and this may be the one of the reason of vaccination failures in last few years (Biswas et al., 2004) reported from India.

Table –I Indian isolates of *Pasteurella multocida* (B:2) , place of isolation, host and ERIC-PCR profiles

Isolate No.	Place of isolation	Host	ERIC-PCR Profile
Isolate 1	Uttar Pradesh	Reference Strain	P1
Isolate 2	Uttar Pradesh	Buffalo	P1
Isolate 3	Uttar Pradesh	Buffalo	P1
Isolate 4	Uttar Pradesh	Buffalo	P2
Isolate 5	Uttar Pradesh	Pig	P2
Isolate 6	MAHARASHTRA	Buffalo	P7
Isolate 7	MAHARASHTRA	Buffalo	P8
Isolate 8	MAHARASHTRA	Buffalo	P2
Isolate 9	MAHARASHTRA	Buffalo	P6
Isolate 10	MAHARASHTRA	Buffalo	P4
Isolate 11	MAHARASHTRA	Buffalo	P5
Isolate12	MAHARASHTRA	Buffalo	P2
Isolate 13	MAHARASHTRA	Buffalo	P1
Isolate 14	MAHARASHTRA	Buffalo	P3
Isolate 15	MAHARASHTRA	Buffalo	P10
Isolate 16	MAHARASHTRA	Buffalo	P10
Isolate 17	Uttar Pradesh	Goat	P11
Isolate 18	Uttar Pradesh	Pig	P12
Isolate 19	Uttar Pradesh	Pig	P12
Isolate 20	MAHARASHTRA	Buffalo	P14
Isolate 21	MAHARASHTRA	Cattle	P15
Isolate 22	DELHI	Buffalo	P14
Isolate 23	ANDRAPPADESH	Cattle	P14
Isolate 24	MAHARASHTRA	Sheep	P14
Isolate 25	MAHARASHTRA	Goat	P14
Isolate 26	MAHARASHTRA	Sheep	P19
Isolate 27	PUNJAB	Buffalo	P20
Isolate 28	PUNJAB	Buffalo	P20
Isolate 29	PUNJAB	Buffalo	P16

Isolate 30	PUNJAB	Buffalo	P18
Isolate 31	MAHARASHTRA	Dog	P17
Isolate 32	MAHARASHTRA	Sheep	P17
Isolate 33	MAHARASHTRA	Cattle	P17
Isolate 34	MAHARASHTRA	Cattle	P21
Isolate 35	MAHARASHTRA	Cattle	P21
Isolate 36	ANDHRAPRADESH	Lion	P21
Isolate 37	MAHARASHTRA	Tiger	P22
Isolate 38	ANDRAPARDESH	Sheep	P13
Isolate 39	MAHARASHTRA	Goat	P13
Isolate 40	TAMILNADU	Sheep	P13
Isolate 41	TAMILNADU	Sheep	P13
Isolate 42	DELHI	Buffalo	P9

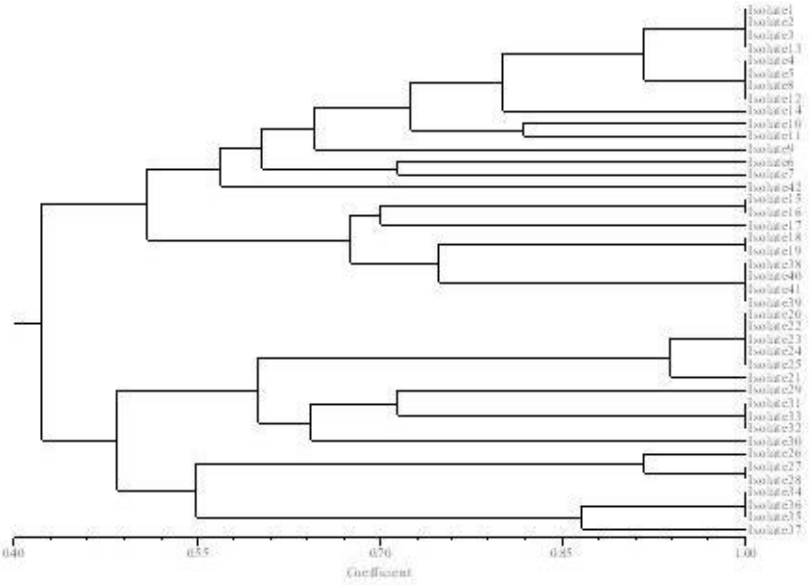

Fig 2: Dendrogram constructed on basis of ERIC-PCR Profiles of isolates of Pasteurella multocida (B:2)

On the basis of our study we presume that a) there is high genetic diversity among serotype B:2 isolates of *Pasteurella multocida* in India b) there is no host or geographic specificity for this serotype, c) a broad host range for this serotype and d) the vaccine strain in use is distantly related to the isolates prevalent in India and e) an urgent need for re-evaluation of vaccine strain in use in India.

Acknowledgments

Director I.V.R.I. and I.C.A.R.(INDIA)are acknowledged for providing funds and necessary facilities for this study.

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DOI:10.22192/ijarbs.2016.03.09.031	

How to cite this article:

M.K. Saxena, A. Kumar, V.P.Singh, P. Chaudhari, S.B. Shivachandra and B. Sharma. (2016). Molecular typing of Indian isolates of *Pasteurella multocida* serotype B:2 by ERIC- PCR. Int. J. Adv. Res. Biol. Sci. 3(9): 232-237.

DOI: http://dx.doi.org/10.22192/ijarbs.2016.03.09.031