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Antibiotic Susceptibility and Molecular Analysis of Bacterial Pathogen *Pasteurella Multocida* Isolated from Cattle

Azmat Jabeen, Mahrukh Khattak, Shahzad Munir*, Qaiser Jamal, Mubashir Hussain
Department of Microbiology, Kohat University of Science and Technology, Kohat, Khyber Pakhtunkhwa, Pakistan.

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ABSTRACT

Pasteurella multocida is a Gram negative, non motile and coccobacillus bacterium. It has 5 strains i.e. A, B, D, E and F and 16 serotypes (1-16). In present study, we analyzed *Pasteurella multocida* B: 2 strains, responsible for Hemorrhagic Septicemia (HS) in cattle, on morphological/microbial, biochemical, molecular level and to check the antibiotic sensitivity of the *Pasteurella multocida*. Microbial analysis showed that while grown on Brain Heart Infusion agar plates and Blood Agar Base Medium, grayish lustrous colonies of *Pasteurella multocida* were observed. Gram staining showed that *Pasteurella multocida* are gram negative. Microscopic observations revealed it to be coccobacillus and it was non- motile. Identification was conducted by conventional biochemical tests and percentage identification of Analytical Profile Index was 96 %. Antibiotic sensitivity with different antibiotics was checked by disk diffusion method and was found resistant to Augmentin, Amoxicillin and Aztreonam and was more susceptible to Ceftiofur. On molecular level its DNA was extracted and was run with marker having range from 0.5 – 10 kb. Its DNA was found heavier than 10 kb. It was concluded that accurate laboratory diagnosis of *Pasteurella multocida* depends on isolation and identification of suspected bacterial colonies by microscopy and biochemical tests. Molecular analysis is a successful tool for differentiation of strains in a variety of bacterial infections.

INTRODUCTION

Pasteurella multocida is an important animal pathogen, particularly causative agent of several economically significant veterinary diseases, which causes fowl cholera in poultry, atrophic rhinitis in swine and haemorrhagic septicaemia in cattle. *Pasteurella multocida* is also a commensal of both human and animal respiratory tract (Ryan and Ray, 2004). *Pasteurella multocida* is a Gram-negative, non-motile, coccobacillus that is penicillin-sensitive and belongs to the family pasteurellacae (Kuhnert and Christensen, 2008). The production of capsular material of *Pasteurella multocida* is affected by sub-minimal inhibitory concentration of antibiotics (Champlin *et al.,* 2002). Complex Outer Membrane Protein (OMP) profile of more than 40 proteins bands was demonstrated in *Pasteurella multocida* isolated from haemorrhagic septicaemia cases. Correlation between the electrophoretic pattern and serotypic properties of isolates were established but no one single protein band could be identified as unique to all strains that caused haemorrhagic septicaemia. Common Outer Membrane Proteins (OMP) bands (27kDa, 34kDa and 36kDa) were common to all isolates regardless to serotype (Bosch *et al.,* 2002). Lipopolysaccharide (LPS) is a major virulence factor and played an essential role in causing diseases as haemorrhagic septicaemia in buffalos. Examination of *Pasteurella multocida* strains from different animals confirmed that LPS from *Pasteurella multocida* were slightly similar to Lipopolysaccharides of Enterobacteriacae. It was reported that Lipopolysaccharide was responsible for the 1-16 somatic serotypes, and when examined electrophoretically the LPS was of low molecular weight. It was confirmed that LPS of *Pasteurella multocida* was shorter than that of Enterobacteriaceae especially those of *Eschericia coli* and *Staphylococcus typhimurium* (Horadagoda *et al.,* 2001). Hemorrhagic septicemia (HS) is an acute disease infecting cattle and buffalo (water & swamp) caused by two specific serotypes of Pasteurella multocida. The Asian serotype is designated B: 2, and the African serotype is E: 2 by Carter-Heddelston system, corresponding to 6: B and 6: E by Namioka-Carter system.

* *Corresponding Author*
Department of Microbiology, Kohat University of Science and Technology, Kohat, Khyber Pakhtunkhwa, Pakistan.
Phone: +923459675779

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The disease is characterized by a rapid course of edematous swelling in the throat and brisket region, swollen and hemorrhagic lymph nodes and the presence of numerous subserous hemorrhages. Hemorrhagic Septicemia is considered economically to be the most important bacterial disease in South-East Asia including Indonesia, Philippines, Thailand, Malaysia, Middle-East, North-East, Central and South Africa (Verma and Jaiswal, 1998). The bacterium spreads from animal to animal by aerosols, and types of diseases it causes in buffaloes are hemorrhagic septicemia and pneumonia (Hirsh, 1989). Thus, enzootic pasteurellosis and epizootic pasteurellosis affect the livestock industry and are responsible for important economic losses in cattle farming (Holmes *et al.,* 1995).Accurate laboratory diagnosis of *Pasteurella multocida* depends on the isolation and identification of suspected bacterial colonies by microscopy and biochemical tests. Samples taken immediately from animals after death yield almost pure cultures of *Pasteurella multocida* e.g. from heart blood, spleen, liver, bone marrow or lung (Townsend *et al.,* 1998). The following antibiotic agents have proven their clinical efficacy on *Pasteurella multocida*: 0penicillin, 0amoxicillin,0 (orampicillin), 0erythromycin,0trimethoprim/sulfamthoxazole,cephalothin,tetracycline,ceftiofur,cefquinome,streptomycin,gentamicin,spectinomycin, florfenicol, sulfonamides, tilmicosin, enrofloxacin (orother floroquinolones) and norfloxacin. The application of the advanced diagnostic techniques as electron microscope investigation, microbial and biochemical or DNA analysis of the *Pasteurella multocida* has helped a great deal in the elucidation of the virulence factors of the organism and their encounter in pathogenesis, as well as help in finding new-targeted generations of antibiotics. Therefore present study was planned to morphologically characterize *Pasteurella multocida* by observing culturing characteristics and by carrying out Gram Staining technique, to identify *Pasteurella multocida* by conducting different biochemical tests, to carry out percentage identification of the *Pasteurella multocida* through Analytical Profile Index (API 20 NE Strip), to check the antibiotic sensitivity of the *Pasteurella multocida* for several antibiotics and to characterize the *Pasteurella multocida* at molecular level by DNA analysis.

METHODOLOGY

The research project was carried out in bacteriology and virology labs of National Veterinary Laboratories, Ministry of Food and Livestock Islamabad.

MORPHOLOGICAL/MICROBIAL ANALYSIS

Isolation from Mice Heart

Pasteurella multocida was isolated from mouse heart preserved at –20°C. That mouse was inoculated with the sample obtained from the cow from Peshawar.

The heart was streaked over Blood Agar Base media and Brain Heart Infusion agar media and those media were incubated for 24 hours at 37°C then growth was observed.

Inoculation into Mice

Some colonies were taken with the help of wire loop in normal saline in test tube, it was shaken and its turbidity was compared with Mac Farland standard.

Colonies were mixed till its turbidity matched with the standard and achieved turbidity of 0.010. Then 8 ml of this normal saline was taken in syringe and injected into 2 mice. One mouse acted as control.

Dissection of Mice

Dissection of mice was carried out and heart was isolated. Streaking was carried out over Blood Agar Base media and Brain Heart Infusion agar and was incubated for 24 hours at 37 °C. Afterwards gram staining was used for enumeration of gram negative bacteria.

BIOCHEMICAL CHARACTERIZATION

Following biochemical tests were carried out for identification and confirmation of *Pasteurella multocida.*

Oxidase Test

On filter paper, Oxidase reagent was poured. With the help of loop, *Pasteurella multocida* was spread over it.

Catalase Test

Some amount of H2O2 was taken in test tube. With the help of wire loop, *Pasteurella multocida* colony was picked up and dipped into H_2O_2.

Indole Test

With the help of loop, colony was picked up and was dipped into the Indole contained in the test tube. It was mixed and incubated for 24 hours.

Triple Sugar Iron Test (TSI)

TSI agar slant was taken. With the help of wire loop, colony was picked up and streaked over the agar slant. It was incubated for 24 hours.

Citrate Test

With the help of straight wire, a colony was picked up and was streaked in a zigzag manner over the slant.

Glucose

With the help of a loop, colony was transferred into glucose test tube. It was rotated inside the test tube for proper mixing. Then it was incubated for 24 hours.

Sugar Test

Tests were carried out with 2% sorbitol; xylose and maltose.1 ml of each was added to glucose media and then incubated for 24 hours.

Nitrate Test

Colony was picked up with the help of a loop and was mixed in nitrate reagent. It was then incubated for 24 hours.1 ml of reagent1 (sulphonic acid) was added and then 1 ml of reagent 2 (alpha-naphthalene) was added.

Motility Test

Motility was checked in peptone water agar. Straight wire loop was passed over flame and a colony was picked up. It was dipped in peptone water agar.

ANALYTICAL PROFILE INDEX (API)

For Analytical Profile Index, API 20NE was used. It is standardized system for the identification of Gram negative rods, combining 8 conventional and 12 assimilation tests**.**

Preparation of Inoculum

18-24 hours old colonies were picked up and were mixed in saline solution to achieve a density of 0.5.It was compared with Mac Farland solution.

Inoculation of the Strip

Inoculums were added from NO_3 to PNPG into tubules but not into the cupules. In the remaining tests inoculum was added up to the cupules. Approximately 200µl of suspension were added from D-glucose to Phenyl Acetic Acid. Mineral oil was added to, D-glucose, Arginine Dihydrolase and Urease up to cupules until a concave meniscus was formed. Distilled water was sprinkled over it and incubated at 37℃ for 24 hours and the result was compared with the table no 1.

ANTIBIOTIC SENSITIVITY TEST

Antibiotic sensitivity was checked by disk diffusion method. Dilution of *Pasteurella multocida* was prepared and it was compared with Mac Farland standard. It was then poured over Muller Hinton agar media and was spread over it. Discs of antibiotics including Gentamycin (10 µg), Cotrimaxazol (10 µg), Aztreonam(30 µg), Augmentin(10 µg), Ceftiofur (10 µg), Amoxicillin +Clavulinic Acid (10 µg), Ofloxacin (30µg), Norfloxacin (5 µg), Cephatoxin (10 µg), were then placed with the help of forceps over the media containing *Pasteurella multocida*. It was then incubated for 24 hours and zones of inhibition were measured.

DNA EXTRACTION

Pasteurella multocida isolates were grown in BHI at 37◦C for 24 hours.1ml cultures was centrifuged in ependrof tube at 13,000 rpm for 4 minutes. Supernatant was discarded and pellets were resuspended in 1ml TE buffer. It was centrifuged again at 13,000 rpm for 4 minutes. All the supernatant was decanted but 50µl of the supernatant was left in the Ependorf tube.150 µl freshly prepared lysozyme solution was added and placed on ice for 10 minutes.40 µl of (10 %) SDS was added and mixed for 1 minute until white suspension was formed.60µl of proteinase K solution was mixed and incubated at 37◦C for 30 minutes.800µl of phenol was added. Centrifuged at 13,000 rpm for 1 minute. 600µl of aqueous phase was transferred to a new ependrof tube containing 150µl of TE. 700µl of 1:1 mixture of phenol and chloroform-isoamyl alcohol was added.425µl of aqueous phase was transferred to tube containing 75µl of 3M sodium acetate and mixed briefly.1ml of ethanol was added and the tubes were inverted several times before placing on ice for 10 minutes. It was then placed on ice for 10 minutes. Centrifugation at 16,000 rpm for 15 minutes was carried out.

Characterization by Using Gel Electrophoresis

0.35 grams of agarose was dissolved in 35 ml of 1x TBE (20ml 10x TBE dissolved in 180ml of DDS water). It was heated and allowed to cool at room temperature. 2.5µl of ethidium bromide was added before solidification of the gel. It was then poured into electrophoresis tray, comb was fixed and the gel was allowed to solidify. After solidification, comb was removed and 1x TBE was added to cover the whole gel. In the next step, 7 µl of DNA was mixed with 3µl of bromo phenol blue and a total volume of 10 µl was loaded into the well. Then current of 30mA and 80V was applied and the sample was allowed to run over the gel. The gel was observed under UV light. Weight of genomic DNA of *Pasteurella multocida* was determined by running along with Sigma marker having range from 0.5-10 kb.7µl of marker was mixed with 3µl of bromo phenol blue and was loaded into one of the well of 1% Agarose gel. Then 7µl of DNA was mixed with 3µl of bromo phenol blue and was loaded into another well. Then current of 30mA and 80V was applied and the sample was allowed to run over the gel. The gel was observed under UV light.

RESULTS AND DISCUSSION

Characterization of Bacteria

Colonies were observed over Brain Heart Infusion agar plates and Blood Agar Base medium after 24 hours of incubation and grayish lustrous, isolated colonies were observed over Brain Heart Infusion agar plates which when observed in light appeared to be bluish from its borders. It was also observed over Blood Agar Base and mucoid colonies were observed as shown in fig 1. It was grown over Mac Conkey plates, however no growth was observed over Mac Conkey agar plates. Heddleston and Gerald in 1975 also observed iridescent, blue and watery mucoid colonies in their study of *Pasteurella multocida.*

Gram staining

The gram staining showed that *Pasteurella multocida* were gram negative and coccobaciillus in shape. The results were in accordance with the result of Masayoshi Itoh *et al*, who in 1980 reported *Pasteurella multocida* as gram negative and oval bacilli. In 2006, Rashida *et al.,* and in 2008 Eukandayo *also* confirmed *Pasteurella multocida* gram negative coccobacilli.

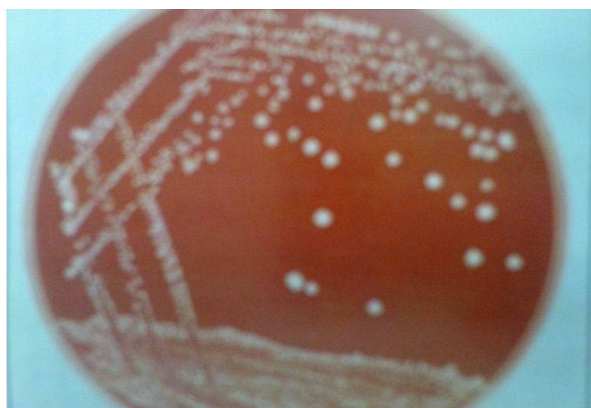

Fig . 1: Growth of *Pasteurella multocida* over Blood agar base medium.

Biochemical characterization

Pasteurella multocida was observed to be Oxidase positive as the enzyme Oxidase oxidizes phenylenediamine. It was observed to be Catalase Test Positive as bubbling appeared in the tube due to production of H_2S gas. Indole Test was also positive, as the oily ring appeared on the surface of the media after Kovacs reagent had been added. Infact in this test the indole was converted into tryptophan. It was TSI positive, as the tube contained bubbles when observed after 24 hours of incubation. Glucose test was observed positive as it carried out fermentation. Sugar test was carried out and was found negative for Sorbitol, Positive for Xylose as it appeared green in color and weakly positive for Maltose as there was slight change in color and appeared fawn in color. The organism was nitrate test positive because red color appeared when inoculated in nitrate broth. The enzyme, nitrate reductase reduce nitrate to nitrite which diazotizes sulphanillic acid. *Pasteurella multocida* was found Citrate negative as it did not require citrate as its sole source of carbon.

It was also found to be urease negative because the enzyme did not hydrolyze media containing urea. Its motility was checked over peptone water agar and this test was found to be negative as *Pasteurella multocida* is non motile. No movement observed in the media. Masayoshi *et al.,* in 1980, also characterized *Pasteurella multocida* biochemically and found similar results. Our result also matched with the result of Rashida *et al.,* who studied biochemical characterization of Pasteurella multocida in 2006.

Analytical profile indexes

The results observed from the API strip was converted into numerical value and the number obtained was 3000004, which was then checked in the catalogue book and *Pasteurella multocida* was identified 96% at this value**.**

In 2006, Rashida *et al.,* however identified *Pasteurella multocida* 93 %.In 2006, Zahoor and Siddique used API kit for the % identification and indicated 86.5 to 96.6% identification of *Pasteurella multocida*.

The results of Analytical profile indexes are shown in table 1.

Antibiotic sensitivity tests

We also checked the antibiotic sensitivity of *Pasteurella multocida* by disc diffusion method. The antibiotics used were amoxicillin, augmentin, cotrimaxazole, aztreonam, ofloxacin, norfloxacin, cephatoxin, gentamycin and ceftiofur and *Pasteurella multocida* was found resistant to Augmentin and Cotrimaxazole and was more susceptible to Amoxicillin and Aztreonam and found sensitive to Gentamycin and Ceftiofur (Table 2). In 2007, Boudewijh also determined antimicrobial susceptibility test by using different antibiotics. He also used gentamycin and ceftiofur in his investigation and also found that *Pasteurella multocida* was sensitive to these two antibiotics.

Table . 1: Result of Analytical Profile Index.

Tests	Active Ingredient	Reactions/Enzymes	Negative Result	Positive Result	Observrd Result
NO_3	Potassium nitrate	Reduction of nitrates to nitrites/nitrogen	Colorless (NIT1/NIT2) Pink (Zn)	Pink red (NIT1/NIT2) Colorless(Zn)	POSITIVE
TRP	L-tryptophane	Indole production TryptoPhane	Colorless,pale green/yellow	pink	POSITIVE
GLU	D-glucose	Fermentation (GLUcose)	blue to green	Yellow	NEGATIVE
ADH	L-arginine	Arginine DiHydrolase	yellow	Orange/pink/red	NEGATIVE
URE	Urea	UREase	yellow	Orange/pink/red	NEGATIVE
ESC	Esculin ferric citrate	Hydrolysis(β -glucosidase) (ESCulin)	yellow	Grey/brown/black	NEGATIVE
GEL	Gelatin (bovine origin)	Hydrolysis (protaess) (GELatin)	No pigment diffusion	Diffusion of black pigment	NEGATIVE
PNPG	4-nitrophenyl-□D-glactopyranoside	β-galactosidase (para-nitrophenyl_ βD galactopyranosidase)	colorless	Yellow	NEGATIVE
GLU	D-glucose	Assimilation (GLUcose)	transparent	opaque	NEGATIVE
ARA	L-arabinose	Assimilation (ARAbinose)	transparent	opaque	NEGATIVE
MNE	D-mannose	Assimilation (ManNosE)	transparent	opaque	NEGATIVE
MAN	D-manitol	Assimilation(MANnitol)	transparent	opaque	NEGATIVE
NAG	N-acetylglucoseamine	Assimilation(N-Acetyl-Glucoseamine)	transparent	opaque	NEGATIVE
MAL	D-maltose	Assimilation(MALtose)	transparent	opaque	NEGATIVE
GNT	Potassium gkuconate	Assimilation (potassium GlucoNate)	transparent	opaque	NEGATIVE
CAP	Capric acid	Assimilation(CAPric acid)	transparent	opaque	NEGATIVE
ADI	Adipic acid	Assimilation(Adipic acid)	transparent	opaque	NEGATIVE
MLT	Malic acid	Assimilation(MaLaTe)	transparent	opaque	NEGATIVE
CIT	Trisodium citrate	Assimilation(trisodium CITrate)	transparent	opaque	NEGATIVE
PAC	Phenylacetic acid	Assimilation (PhenylAcetic Citrate))	transparent	opaque	NEGATIVE
OX		Cytochrome Oxidase	transparent	opaque	POSITIVE

Table. 2: Result of Antibiotic Sensitivity Test for *Pasteurella multocida.*

Antibiotics	Abbreviation	Concentration µg	Zone of Inhibition Mm	Sensitive/ Resistant
Gentamycin	CN	10	25	Sensitive
Cotrimaxazol	ACC	10	24	Sensitive
Aztreonam	ATM	30	Nil	Resistant
Augmentin	AMC	10	Nil	Resistant
Ceftiofur	SXT	10	39	Sensitive
Amoxicillin +Clavulinic Acid	AMC	10	Nil	Resistant
Ofloxacin	OFX	30	26	Sensitive
Norfloxacin	NOR	5	31	Sensitive
Cephatoxin	CTX	10	22	Sensitive

Genomic DNA isolation

Last step was the isolation of genomic DNA by enzymatic lysis and its weight determination. Pellet was obtained and run over gel and then observed in UV light. Glowing band was observed and was also run with a marker of 0.5- 10 kb. DNA was found more than 10 kb. In 2000, Richard and Rimler also isolated chromosomal DNA of *Pasteurella multocida* by enzymatic method.

CONCLUSIONS

Pasteurella multocida is an important animal pathogen and particularly cause diseases in several economically significant animals and it is a commensal of both human and animal respiratory tract. Infection caused by *Pasteurella multocida* is diagnosed or identified through different microbial, biochemical and molecular techniques. We characterized *Pasteurella multocida* in our research project at morphological/microbial, biochemical and molecular levels. It was concluded that accurate laboratory diagnosis of *Pasteurella multocida* depends on isolation and identification of suspected bacterial colonies by microscopy and biochemical tests. Molecular analysis is a successful tool for differentiation of strains in a variety of bacterial infections.

REFERENCES

Bosch, M., Garrido, M.E., Perez De Rozas, A.M., Badiola, I., Barbe, J. Characterization of *Pasteurella multocida* hgbA gene encoding hemoglobin binding protein. Infect. Immun. 2002; 70(11): 5955-5964.

Champlin, F.R., Shryock, T.R., Patterson, C.E., Austin, F.W., Ryals, P.E. Prevalence of a novel capsule-associated lipoprotein among pasteurellaceae pathogenic in animals. Curr Microbial. 2002; 44(4): 297-301.

Ekundayo, S.O., Odugdo, M.O., Olabode, A.O., Okewole, P.A. Phenotypic variability of *Pasteurella multocida* isolated from avian,bovine,leporine and ovine origin. Afr. J biotech. 2008; 7:1347-1350.

Hirsh, C., Hansen, L.M., Dorfman, L.C., Snipes, K.P., Carpenter, T.E., Hird, D.W., McCapes, R.H. Resistance to antimicrobial agents and prevalence of R plasmids in *Pasteurella multocida* from turkeys. Antimicrob Agents Chemother. 1989; 33:670–673.

Heddleston, K.L. Characteristic of *Pasteurella multocida* of human origin. J. Clin. Micro. 1975; 1:377-383.

Holmes, B., Pickett, M.J., Hollis, D.G. Unusual gram-negative bacteria, including *Capnocytophaga*, *Eikenella*, *Pasteurella*, and *Streptobacillus*. Manual of clinical microbiology. 6th ed. Washington, D.C (1995) 499–508.

Horadagoda, N.U., Hodgson, J.C., Moon, G.M., Eckersall, P.D. Role of endotoxins in the pathogenesis of haemorrhagic septicaemia in buffalo. Microb. Pathol. 2001; 30 (3): 171-178.

Kuhnert. P., Christensen, H. Pasteurellaceae: Biology, Genomics and Molecular Aspects. Caister Academic Press. 2008; 34-39.

Masayoshi, I., Tierno, P.M., Milstoc, M., Berger, A.R. A unique outbreak of *Pasteurella multocida* in a chronic disease hospital. Ameri. J. pub. Health. 1980; 70:1170-1173.

Mohammad, Z., Siddique, M. Characteristics of *Pasteurella multocida* recovered from avian sources. Pak. Vet. Journal. 2006; 26:41-43.

Rashida, M., Farooq, U., Bader, N., Khanum, A., Wadood, A. Restriction endonuclease analysis of *Pasteurella multocida* field isolates by Hha-1.Pak. Vet. journal. 2006; 26:80-84.

Rimler, R., Richard, B.R. Restriction endonuclease analysis using Hhal and Hpall to discriminate among group B *Pasteurella multocida* associated with haemorrhagic septicaemia. J. Med. Microbio. 2000; 49: 81-87.

Ryan, K.J., Ray, C.G. Sherris Medical Microbiology, 4th ed. McGraw Hill. (2004) 8529-85399.

Townsend, K., Front, A.J., Lee, C.W., Dwakins, H.J. Development of PCR assays for species and type-specific identification of *Pasteurella multocida* isolates. J. Clinical. Micro. 1998; 36: 1096–1100.

Verma,R., Jaiswal, T.N. Hemorrhagic septicaemia vaccines. Vacc. 1998;16: 1184–1190.

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