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POLYPEPTIDE MAPPING AND WESTERN BLOT ANALYSIS OF *MYCOPLASMA GALLISEPTICUM* FIELD ISOLATES

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ABSTRACT

Mycoplasma gallisepticum (MG), an intracellular bacterium, causative agent of chronic respiratory disease (CRD) of poultry, is transmitted vertically and horizontally. MG is cosmopolitan in distribution and causes huge economic losses to poultry industry. To lower economic losses and spread of bacteria, molecular characterization can provide basis for its control. Field isolates of MG prevalent in Pakistan were characterized to check antigenic variations and determination of immunogenic proteins. Protein profiling was carried out using Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) using 12.5 percent resolving gel under reduced conditions. Determination of immunogenic antigens among segregated proteins was done by western blotting. Five major polypeptides with molecular weights of 32.35, 43.65, 52.48, 64.56 and 70.8kDa were revealed. No antigenic variation was observed by SDS-PAGE among isolated bacteria from out breaks of CRD. Out of five polypeptides three with molecular weights of 52.48, 43.65 and 32.35kDa were declared immunogenic in nature as determined by western blotting. Results of present study can be used as base line for production and evaluation of subunit vaccines.

Key words: *Mycoplasma gallisepticum*, CRD, SDS-PAGE, Polypeptide and Western blot.

INTRODUCTION

In Pakistan, poultry industry is playing pivotal role to cope up food demand of human population. Industry has contributed 23.8 percent of total meat and 13144 millions eggs to meet the need of protein as reported in Economic Survey of Pakistan (2012). Profitability directly correlates with the good management and rearing disease free flocks. Poultry industry in Pakistan is facing problems due to large number of infectious and non infectious maladies. Among infectious diseases Chronic Respiratory Disease (CRD) is at top rank causing economic losses to poultry (OIE, 2011).

CRD is induced by *Mycoplasma gallisepticum (MG)* which is the smallest, wall less, intracellular prokaryote (Rottem *et al*., 2003). MG has ability to evade from host's immune system and consequently persist for longer period. It characteristically localizes in reproductive tract of laying birds and is vertically transmitted (Yoder and Hofstad, 1964). Infected birds remain carrier and become a constant threat to healthy poultry population (Saad and Dirgham, 2008).

Proper diagnosis is essential in order to design effective strategies for timely treatment and eradication of disease (Bencina *et al.,* 2003). Clinical signs and necropsy findings are usually confusing with many other respiratory diseases. Due to fastidious nature of bacteria, isolation trials of MG are usually unsuccessful, quite difficult, time consuming and laborious (Kleven, 2003).

Among serological tests, the rapid plate agglutination (RPA) test is very simple, sensitive and used for the detection of MG antibodies under field conditions (Biro *et al.,* 2006). For more specific and sensitive diagnosis of MG Enzyme Linked Immunosorbant Assay (ELISA) can be used (Ley, 2003; Ahmad *et al*., 2008). Molecular techniques are preferred over all other methods of disease diagnosis (Hagen *et al*., 2002). Sodium dodecyl sulphate polyacylamide gel electrophoreses (SDS-PAGE), a molecular technique, is used to identify proteins related to virulence and phenotypic diversity of different strains of MG (Papazisi *et al*., 2002). Western blotting detects antibody response, by reaction of antigen and antibody on nitrocellulose membrane (Thomas and Sharp, 1990). Main aim of this integrated approach is early and accurate diagnosis of MG and rapid curtailing the spread of disease. Ultimately goal was to lower economic losses to commercial poultry industry.

MATERIALS AND METHODS

The present study was conducted on layer birds suffering from Chronic Respiratory Disease (CRD). *Mycoplasma gallisepticum* (MG) was isolated and identified using conventional biochemical tests (OIE, 2011). MG isolates (n=12) were used for proteomics analysis and determination of immunogenic polypeptides. Sodium dodecyl sulphate polyacralimide gel electrophoresis (SDS-PAGE).

Different proteins of MG isolates were analyzed by SDS-PAGE following standard protocol using discontinuous buffer system under reduced conditions (Laemmli, 1970; Ferraz *et al.*, 2000; Jan *et al.*, 2001). Activated MG broth culture (1.5mL) was centrifuged at 12000rpm for twenty minutes. Pellets were dissolved in phosphate buffered saline (PBS) and sonicated by three shots 30 seconds with an interval of 10 seconds by placing in ice jar. Sonicated suspension was centrifuged at 3000rpm for 20 minutes, supernatant mixed with 2X denaturation buffer (Stacking gel buffer: 2.5ml, 10% SDS: 4ml, Glycerol: 2ml, β-mercaptoethnol: 1ml, Bromophenol blue: 2 drops and water up to 10ml) at 1:1 ratio and boiled in water bath at 100^0C for 3 minutes. Polyacrylamide resolving gel (12.5%) was prepared by mixing 30% monomer acrylamide stock (10ml), resolving gel buffer (7.5ml), 10%SDS (0.3ml), 10%APS (15µl), TEMED (10µl) and finally distilled water was added to make 30 ml total volume. Gel mixture was poured in vertical glass casting plates and allowed to polymerize for 30 minutes. Polyacrylamide stacking gel (4%) was prepared by mixing 30% monomer acrylamide stock (1.33ml), Stacking gel buffer (2.5ml), 10%SDS (0.1ml), 10%APS (50µl), TEMED (5µl) and finally distilled water was added to make 10 ml total volume. Gel mixture was layered over separating gel and comb was inserted for well formation.

Prepared MG samples (50ul) were loaded in independent wells of stacking gel along with known protein markers having protein molecular weight range 14.4 to 116 kDa (Bio-Rad). Casting plates were placed in electrophoresis chamber having running buffer (Tris base 12g, Glycine 57.6g, 10%SDS 40ml and distilled water up to 4 liter; pH: 8.8).

Electrophoresis was performed at constant current supply (100V). Gels were stained by Commassie Brilliant Blue (1% stock stain: 62.5ml, methanol: 250ml, glacial acetic acid: 50ml and distilled water up to 500ml) for four hours. Later on, gels were destained (Methanol: 250ml, glacial acetic acid: 50ml and distilled water 200ml), photographed and relative flow (R_f) value for each polypeptide was calculated dividing distance travelled by polypeptide over total distance travelled by tracking dye (Bromophenol blue).

A standard calibration curve was plotted between R_f values and log molecular weights of segregated protein marker. Polypeptides of samples were determined by plotting R_f values on standard curve and calculating molecular weights from log molecular weight values.

Western Blotting: Western blot on segregated MG polypeptides was carried out according to standard procedure as described by Ellakany *et al.* (1997). Proteins of MG separated in resolving gel were transferred to nitrocellulose membrane using mini blot apparatus. Gels and nitrocellulose membrane were tightened between sandwich clamps and placed in running tank containing transfer buffer (25mM Tris base: 2.025gm/lit, 192mM Glycine: 14.4gm/1000ml, Methanol 20% V/V: 200ml and distilled water was added to make final volume 1000ml).

Current (30V) was supplied overnight for transfer of segregated proteins from polyacrylamide gel to nitrocellulose membrane. Membranes were blocked with horse serum (5%) and bovine serum albumin (3%) in tris buffer saline at 37^oC for an hour. Membranes were incubated with antibodies raised against MG in rabbits following the procedure described by Senterfit (1983) for four hours. Alkaline phosphatase labeled anti-rabbit antibodies (IgG) were poured on nitrocellulose membrane and incubated for an hour at room temperature. Membranes were washed and p-nitrophenyl phosphate (substrate) was poured on for 15 minutes for development of color by reaction of enzyme and finally reaction was stopped by acid solution. Gels were dried, photographed and immunogenic proteins of MG isolates were determined. Data was analyzed statistically using chi square test at probability level of 0.5.

RESULTS AND DISCUSSION

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is routinely used for proteomics analysis of different microbes. In present study *Mycoplasma gallisepticum* (MG) field isolates were characterized to observe the differences and similarities in polypeptide pattern. A standard calibration curve was plotted between log molecular weight of known protein markers and their relative flow (R_f) values. Molecular weights of MG polypeptides were calculated from this curve. Protein markers with molecular weights, log molecular weights and R_f values are shown in table (01). Most of the MG isolates revealed five polypeptides in resolving gel on electrophoresis and R_f values, log molecular weights and molecular weights of these polypeptides are presented in table (02).

All of the field isolates of MG were similar in polypeptide pattern. Five polypeptide bands with molecular weights of 32.35, 43.65, 52.48, 64.56 and 70.8kDa were detected by SDS-PAGE (plate 01). Protein bands of 43.65kDa represented surface protein as documented by Gorton and Geary (1997) detected in S6 strain of MG. Membrane proteins with molecular size of 18, 37, 38, 41, 43 and 91kDa were revealed in different phenotypes of MG. Similarly Buyuktanir *et al*. (2008) reported 44kDa as surface protein specific to MG in accord to the present findings. Polypeptide detected with molecular weight of 32.35kDa was same as reported by Linda *et al.* (1998). Protein of 32.6kDa was identified as cytadhesin-like protein MGC2. The results of present study were in close conformity with finding of Jan *et al*. (2001).

Results of present study are comparable with Collett and Thomson (2005) observations. It was concluded that similar antigenic components are presented in all of the field isolates of MG on protein profiling and in corroboration with findings of Ferraz *et al*. (2000). SDS-PAGE can effectively be used to study pattern of proteins in field MG isolates and imported vaccine strains for homology. Any change in polypeptides of MG field isolates can be detected efficiently.

Out of five polypeptides only three were exhibited by western blotting on nitrocellulose membrane. Polypeptide bands visualized by western blot were of 52.48, 43.65 and 32.35kDa molecular weights. Thomas and Sharp (1990) detected carbohydrate-containing components by periodic acid-Schiff stain in three out of five strains of MG. Difference was noticed by PAS, electrophoretic mobility, numbers and lectin reactive bands in five different strains of MG.

Ellakany *et al*. (1997) examined sensitivity of western blot in experimentally infected birds. Proteins related to virulence of MG strains were identified using western blot by Papazisi *et al*. (2002). Proteins were identified as GapA, cytadhesin molecule, the CrmA putative cytadhesin-related molecule, and HatA a component of a high-affinity transporter system. Both CrmA and GapA are essential for MG virulence was suggested. Westren immunoblot were used to confirm infection status of birds that had discrepancies between HI and ELISA (Ewing *et al.*, 1996). Surface proteins P67 (pMGA) and P52 of MG were analyzed by Jan *et al*. (2001). Results indicated that P67 is a true membrane-associated lipoprote pMGA1.2 and P52 is not lipoprotein. Results of immunoblotting were that surface protein P52 and P67 were specific to MG.

May *et al*. (2006) and Buyuktanir *et al*. (2008) confirmed MG proteins related to virulence efficiently by western blotting. Proteins of MG vaccine-type (F-K810) and wild-type S6 (208) strains cultured in Hay flick's media were analyzed through SDS-PAGE by Ferraz *et al*. (2000). There was no visual change in protein profile of those strains but peptide band of p75 was specifically observed only in F-K810 strain. Gorton and Geary (1997) determined expressed proteins of MG isolates and membrane proteins of 91, 43, 41, 38, 37, and 18 kDa were revealed in variant phenotype but not in parental phenotype.

It was concluded that there was little antigenic variation among different strains of MG prevalent in Pakistan and SDS-PAGE along with western blotting can be used for molecular characterization of MG isolates.

Table 01: Protein markers of known molecular weights and their respective R_f-values determined by SDS-PAGE

Sr.#	Protein Marker	Molecular weight (kDa)	Log Molecular weight	R_f-Value
1	β-galactosidase	116.0	2.064	0.141
2	Bovine serum albumin	66.2	1.82	0.203
3	Ovalbumin	45	1.65	0.256
4	Lactate dehydrogenase	35	1.544	0.300
5	REase Bsp981	25	1.39	0.352
6	β –lactoglobulin	18.4	1.29	0.404
7	Lysozyme	14.4	1.16	0.448

R_f: Relative flow, SDS-PAGE: Sodium dodycyl sulphate polyacrylamide gel electrophoresis

Table 02: R_f values, log molecular weights and molecular weights of M*ycoplasma gallisepticum* field isolates polypeptides determined by SDS-PAGE

Sr.#	R_f value	Log Molecular weight	Molecular weight
1	0.19	1.85	70.8
2	0.12	1.8	63.09
3	0.243	1.7	50.11
4	0.263	1.65	45
5	0.297	1.54	34.67

R_f: Relative flow, SDS-PAGE: Sodium dodycyl sulphate polyacrylamide gel electrophoresis

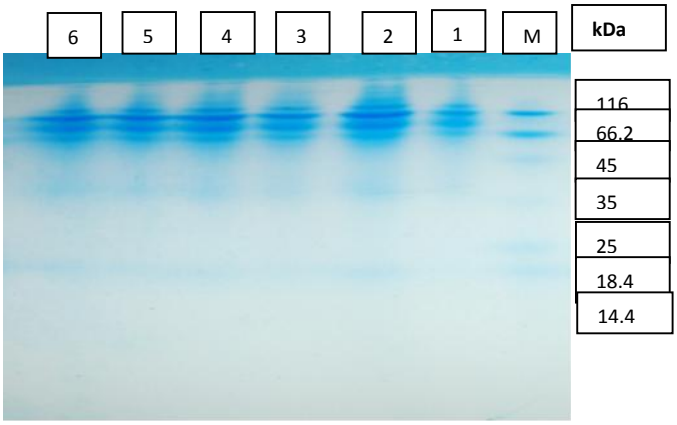

Plate 01: polypeptide map of *Mycoplasma gallisepticum* field isolates revealed in 12.5% separating gel by SDS-PAGE

M: Protein marker, 1-6: Field isolates and kDa: Kilo Dalton

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