ANALYSIS OF PROTEIN PATERNS
OF PASTEURELLA MULTOCIDA STRAINS
ISOLATED FROM POULTRY

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The proteins of 18 field Pasteurella multocida strains isolated from poultry and 3 reference strains were separated electrophoretically at a 10% gradient on polyacrylamide gel in SPD-PAGE. The strains belonged to serotype 1 or 3 according to Heddleston. The separation showed 18 to 31 clearly visible protein bands with various molecular weight. The densitograms of the examined strains were compared. The densitometric analysis permitted finding some qualitative and quantitative differences in the protein electrophoretic profiles of selected isolates. The highest differences in molecular weights and optical density of protein bands were noted in the 32 kDa to 38 kDa range. Immunogenic proteins were detected by immunoblotting using antiserum from chickens immunized with whole-cell antigens of serotypes 1 and 3.

Key words: poultry, Pasteurella multocida, protein, PAGE, immunoblotting.

Fowl cholera is an infectious, world-wide spread disease, caused by Pasteurella multocida. Studies performed in the Department of Microbiology, National Veterinary Research Institute revealed the biochemical and serological characterization of 123 P. multocida strains isolated from poultry (1, 8, 9, 15, 16). It was found that the capsular antigen type A occurred in 84 isolates (68.3%) and the antigen type D was evidenced in 2 strains. Out of 123 P. multocida strains, 82 belonged to serotype 1, 32 to serotype 3 and 8 to seldom occurring serotypes; 6, 8, 10 and 14 (16).

Numerous authors have paid increasing attention to the molecular structure of outer membrane proteins. These proteins may serve as virulence factors (5, 6). Moreover, they are involved in adhesion to the epithelial cells of the respiratory system (13), resistance processes of the bacteriocidal activity of serum, and resistance to phagocytosis (18). Several outer membrane proteins are immunogens and the antibodies against them demonstrate a strong protective activity. Such antigens may be used as the components of subunit vaccines (3). The separation of these proteins using polyacrylamide gel electrophoresis in the presence of SDS is one of the methods of the analysis of surface structures of P. multocida strains.

The aim of the study was to determine and to compare electrophoretic profiles of the proteins of P. multocida strains isolated from poultry and belonging to serotypes 1 and 3 and those of the reference strains using SDS-PAGE. Additionally,
immunogenic proteins transferred to a nitrocellulose membrane were detected using immunoblotting.

Material and Methods

Bacterial strains. Eighteen inland *P. multocida* strains isolated from fowl cholera affected poultry in 1982 to 1997 were examined; 9 isolates belonged to serotype A:1 (P90, P115, P118, P127, P166, P167, P197, P214, and P216), 8 isolates belonged to serotype A:3 (P109, P134, P151, P153, P159, P188, P200 and P203) and 1 to serotype A:3,4 (P228). Three reference strains: TS8 (serotype A:1) obtained from Dr Namioka (National Institute of Animal Health, Tokyo), and X-73 (serotype A:1) and P1059 (serotype A:3) from the National Animal Disease Laboratory, Ames, Iowa, USA, were also examined.

Preparation of *P. multocida* strains for SDS-PAGE. Twenty-one *P. multocida* strains cultured for 18 h on DSA medium (Difco) were washed twice with sterile PBS. Then, the mean suspension density of each strain (% T – 2.0, Abs. 2.0) corresponding to $5 \times 10^9$ cfu/mL was evaluated spectrophotometrically using a Pye-Unicam SP-8-100 UV/Vis apparatus at a 540 nm wave length. Each suspension was mixed with the same volume of a sample buffer with 15% ß-mercaptoethanol containing 6% of SDS. The mixture was incubated for 1 h at room temperature and kept frozen at –20°C until use.

SDS-PAGE. The electrophoretic separation was carried out in 10% polyacrylamide gel according to Laemmli (11). The Low Range of SDS-PAGE Molecular Weight Standards (Bio Rad) were separated on each gel in two extreme holes. The separation was carried out at 100 V voltage, at room temperature for about 1 h and 50 min until the dye front (bromophenol blue) was 1 mm to 2 mm before the end of gel. Gels were stained with Coomassie blue and then analysed using Quantity One program (Bio Rad). The analysis of the examined strains involved molecular weight, optical density, the percentage of protein fractions and the rate of the electrophoretic profile homology.

Immunoblotting. It was performed after Towbin et al. (19) using sera diluted 1:200 from the chickens immunized with whole-cell antigens obtained from two reference strains: TS8 (serotype 1) and P1059 (serotype 3) according to the method of Heddleston (9). Anti-Chicken IgG peroxidase conjugate (Sigma) was applied; 4 chloro-1-naphthol (Sigma) served as a substrate.

Results

The electrophoretic patterns of *P. multocida* proteins of serotypes 1 and 3 were shown on the 10% polyacrylamide gel (Figs 1 and 2). The electrophoretic profiles of the strains of serotypes 1 and 3 were similar. However, the densitometric analysis allowed us to find quantitative and qualitative differences between the strains and serotypes. The electrophoregrams evidenced the presence of 18 to 31 protein fractions of about 24 to 140 kDa. The highest differences in molecular weights and optical density of protein bands were noted in the 32 kDa to 38 kDa range.
Fig. 1. Electrophoretic profiles of the proteins of \textit{P. multocida} strains of serotype 1: P166 (2), TS8 (3) and P216 (4), and serotype 3: P153 (5), P1059 (6) and P134 (7). Lines 1 and 8 comprise the weight standards. Samples were analysed on 10% polyacrylamide gel.

Fig. 2. Electrophoretic profiles of the proteins of \textit{P. multocida} strains of serotype 1: P167 (2), X-73 (3) and P115 (4), and serotype 3: P151 (5) and P159 (6), and serotype 3,4 – P228 (7). Lines 1 and 8 comprise the weight standards. Samples were analysed on 10% polyacrylamide gel.
Fig. 3. Detection of the proteins of *P. multocida* strains of serotype 1: P197 (2), TS8 (3), P214 (4), X-73 (5) and P90 (6) and serotype 3 - P1059 (7) by the sera of a chicken immunized with the strain P1059. Lines 1 and 8 comprise the weight standards. Samples were analysed on 10% polyacrylamide gel.

Examination of 2 reference strains of serotype 1 demonstrated the presence of 18 to 26 clearly visible protein bands. Fractions with molecular weights of 140, 120 to 125, 94 to 98, 82 to 84, 72 to 74, 66, 64, 58 to 60, 52, 48 to 50, 42, 37, 34, 30 to 31, 28, and 26 in kDa were found. The highest optical density was found in protein fractions of 52, 48 to 50, and 37 in kDa. The electrophoretic profiles of X-73 and TS8 strains revealed a 79.9% homology.

The densitometric analysis of serotype 1 isolates showed the presence of 21 to 28 protein bands; fractions of 140, 104 to 106, 94 to 98, 82 to 84, 70 to 74, 66, 64, 60, 52, 50, 48, 40 to 42, 36 and 37, 33 and 34, 30 and 31, 28, 26, and 24 in kDa were mainly noted. The highest OD value showed 50 kDa and 52 kDa bands. In the 32 kDa to 37 kDa range, the majority of the strains showed the most intense colouring for the protein of 36 to 37 kDa and in the case of 3 strains for 33 kDa to 34 kDa (P90, P214, P216). An additional band of the molecular weight of 35 kDa was found in 4 strains (P115, P166, P167, and P214); this protein of P115 isolate revealed the highest optical density. On the other hand, P90, P115, and P197 isolates had also the 32 kDa protein. The electrophoregrams of the field strain of serotype 1 demonstrated a homology rate between 43.9% to 82.5% in comparison to that of the reference strain TS8.

The SDS-PAGE separation of the proteins of the reference strain P1059 of serotype 3 showed bands corresponding to 140, 130, 100, 88, 82, 74, 66, 64, 58, 52, 50, 48, 42, 37, 35, 33, 30, 29, and 26 in kDa. The proteins in the 52, 50, and 37 (in kDa) regions were stained most intensely.
The densitometric analysis of the electrophoretic profiles of the isolates of serotype 3 permitted distinguishing 23 to 31 protein fractions showing various optical density. The occurrence of common bands of 140, 125 to 130, 100 to 104, 86 to 88, 80 to 82, 72 to 76, 66, 64, 58 to 60, 52, 50, 48, 42, 37 to 38, 34 to 45, 32 to 33, 30 to 31, 28 to 29, and 26 in kDa was noticed. All the strains showed a high OD value in 50 kDa and 52 kDa. In the 30 kDa to 38 kDa range the proteins of 3 isolates showed 4 bands (37 to 38, 36, 34 to 35, and 32 to 33 in kDa); however, P203 and P228 strains demonstrated 2 protein fractions with 34 to 35 kDa, and 32 to 33 kDa. The most intensely coloured bands 37 kDa to 38 kDa, 36 kDa, 32 kDa to 33 kDa were found in P109 and P153, P151 and P159, and P203 isolates, respectively, and the remaining strains for molecular weights of 34 kDa to 35 kDa. The electrophoretic pictures of the field isolates of this serotype showed a 56% to 79.8% homology in relation to that of the reference strain P1059.

Further examinations involved the proteins transferred to the nitrocellulose membrane and the detection of antigens using chicken sera after immunization with whole-cell antigens of serotypes 1 and 3 (Fig. 3). Two sera detected the proteins represented by 11 to 18 protein bands separated in SDS-PAGE.

The densitometric analysis of the proteins in the examined strains by the sera specific for the two serotypes demonstrated that these sera reacted with proteins revealing the molecular weights of 104 to 106, 94 to 98, 70 to 74, 64, 60, 52, 50, 36 to 37, 33 to 34, and 30 to 31 in kDa.

**Discussion**

Our results support the data reported by other authors (2, 10, 12) who failed to find electrophoretic profiles typical for serotypes. Ireland et al. (10) analysed protein profiles of *P. multocida* strains of serotype 1 isolated from fowl cholera cases. They found that the protein profiles of sonicated isolates were complex with a large number of bands. The patterns obtained with Commassie blue staining of soluble protein extracts were similar. The major differences among isolates were seen in the 34 kDa to 35 kDa region.

Experiments carried out by Lee et al. (12) with the use of 21 strains belonging to serotypes 1, 4, 3, 3, 4, and 10 sampled from turkeys and chickens demonstrated that the membrane protein patterns were similar in all the strains. The researchers found the presence of a total of 26 bands ranging in molecular weight from greater than 200 kDa to 14 kDa. Most bands were common in all the isolates. Variation was seen in the 30 to 40 kDa range where differences in migration and number of major bands were noted. Up to three bands were present; they varied in intensity with each strain. No serotype-specific pattern was detected.

Choi et al. (2) showed that field strains of serotype A:3 differed in outer membrane protein profiles and did not show outer membrane proteins which were identical to those found in the reference strain P1059.

The immunoblot analysis with the use of the sera against the whole-cell antigens of serotypes A:1 and A:3 in 21 *P. multocida* isolates permitted us to detect immunogenic proteins with various molecular weights.

Dawkins et al. (4) found at least 17 antigenic bands from 14.2 kDa to 96 kDa in immunoblotting with the use of the sera of the mice immunized with the vaccines prepared from the *P. multocida* M14014 strain.
Numerous researchers found that *P. multocida* strains multiplied *in vivo* and *in vitro* on iron-restricted media produce surface antigens revealing protein properties and high molecular weights. These antigens are called iron-regulated outer membrane proteins (14, 17). This method of culturing was used to prepare the antigens of subunit vaccines with advantageous immunogenic properties (2, 3, 7). The subject of further studies will involve determination of electrophoretic profiles of the outer membrane proteins of the two serotypes isolates obtained on media with or without iron chelate (2,2'-dipyridyl) and their use in chicken vaccination.

Summing up, we separated proteins electrophoretically in SDS-PAGE and analysed densitometrically electrophoregrams of the *P. multocida* strains of serotypes 1 and 3 isolated from poultry. Moreover, these proteins were detected in immunoblotting using chicken sera after immunization with the whole-cell antigens of the two serotypes.

**References**


