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Differentiation of Closely Related Isolates (vaccinal strains) of *Pasteurella multocida* using FT-IR

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The present study was undertaken to differentiate closely related vaccinal strains (B: 3, 4 and B: 6) of Pasteurella multocida. Observations were made on the biochemistry of whole cell, envelope and OMPs of using analytical technique, FT-IR. The spectral peaks of whole cells, envelope and OMPs were differentiated between B: 6 and B: 3, 4 by FT-IR analysis. Spectral peaks of B: 3, 4 whole cells were at 644 cm–1 and at absorbance of 5.90, while B: 6 spectral peaks were observed at wave numbers 628, 640 and 651 cm–1 and absorbance at 0.35, 0.66 and 0.67 respectively. Peaks for B: 3, 4 envelope preparations were at 646 wave number (cm-1) and at absorbance of 0.75 but in B: 6 envelope they were at 628, 640 and 651 wave number (cm-1) and at absorbance of 0.35, 0.43 and 0.24 respectively. The spectral peak was found in B: 3, 4 OMPs at 646 wave number (cm-1) and at absorbance of 5.90, while spectral peaks for B: 6 OMPs were at 628, 640 and 651 wave number (cm-1) and at absorbance of 0.60, 0.50 and 0.21 respectively.

It was concluded that the technique applied in this study was useful for better understanding of the structural configurations of the organism. The data generated will help in development of good vaccines which will help in control of HS disease in cattle, detailed studies with a large number of isolates is warranted.

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KEYWORDS
FT-IR (Fourier Transform Infra red spectroscopy), OMPs (Outer membrane proteins), LPS (Lipopolysaccharides).

INTRODUCTION

Haemorrhagic Septicemia (HS) is a particular form of Pasteurellosis caused by *P. multocida* and manifested by an acute and highly fatal septicemia mainly in cattle and buffaloes (2). The disease is caused by two specific serotypes of *P. multocida*. Serotypes B:2 is more frequently found in Asia and E:2 is more frequent in Africa (4). These serotypes are designated as 6: B and 6:E by (11) and (1).

Most strains of Pasteurella form a polysaccharide capsules or envelope. It is composed of polysaccharides, Lipopolysaccharides and variety of protein (8). Capsulation does not affect the LPS profile (14). Proteins and lipids are present among the network of polysaccharides. Both LPS and polysaccharides play a role in passive haemagglutination. (13) described the three antigen complexes alpha, beta and gamma polysaccharides protein complex, a serogroup-specific polysaccharide and LPS. (10) observed that the immunogenicity of certain LPS preparation was due to the presence of contaminating OMPs.

The outer membrane protein (OMP) of Gram negative bacteria has a role in disease processes as it acts at an interface between the host and

pathogen (9). Outer membrane proteins (OMPs) of *P. multocida* plays significant role in the pathogenesis of pasteurellosis and have been identified as potent immunogens. Thus a variation in OMPs profile among the isolates may help in epidemiological survey. Iron is an essential element for most organisms due to its role in metabolic electron transport chains. However, the host iron is largely unavailable due to the presence of iron binding glycoprotein such as transferrin and lactoferrin. Pathogenic bacteria such as *P. multocida* overcome this in vivo by expressing a number of iron regulated proteins and low molecular weight siderophores which sequester iron from iron binding host proteins. Iron regulated proteins include outer membrane proteins, which are involved in the iron acquisition and other virulence factors such as toxins and haemolysins (3).

FT-IR: Fourier transform infrared (FT-IR) spectroscopy has been applied as a rapid, nondestructive, and physico-chemical method for discrimination, classification, and identification of intact microbial cells. Since the identification of pathogenic bacteria using an FT-IR method was reported by (12) .Fourier transform infrared (FT-IR) spectroscopy has been used to characterize and differentiate intact microbial cells based on the biochemical heterogeneity of outer membrane cell components such as lipoproteins, Lipopolysaccharides (LPS), and phospholipids (12). Infrared (IR) spectra depend on the vibrational energy of the molecules in a sample, and spectra of intact bacteria contain a superposition of many specific bands due to the complex nature of cell membranes (6). Therefore, the use of multivariate statistical analysis (or chemometrics) is necessary to obtain qualitative or quantitative information from complex spectra collected in the entire mid-IR spectral range of 4000–400 cm-1 OMP extracts from bacterial cell membranes could exhibit distinctive spectra in the amide I/II region, preventing superimposed absorbance bands from other cellular components (i.e., lipids or carbohydrates) in intact cells. The use of OMP spectra combined with chemometrics could be advantageous over using spectra of intact cells for classification and differentiation of micro-organisms.

The present research focuses on study the biochemistry of whole cell, envelope and OMPs of vaccinal strains using analytical technique, FT-IR.

MATERIALS & METHODS

The samples were prepared by diluting the sample 1:10 in distilled water. Its Optical density was adjusted to 0.1 at the wavelength of 520 nm using spectrophotometer (unico 1100rs). The samples thus prepared were subjected to the FT-IR (Nicolet Nexus 670 FT-IR) analysis for generation of spectra. The germanium crystal of sample loading point was first cleaned with the help of alcohol and then the sample of 10µl poured onto that germanium crystal and adjusted to make ready for measurement. At each and every step before addition of the sample, the background spectra were taken which was the most important step in FT-IR spectrophotometery. The already fed software OMNIC FT-IR help generated the spectra of the sample. During generation of the spectra, the FT-IR auto gains the sample and generates spectra.

The spectra were generated by the four sources;

- Power which provides the current,
- Scan which scans the image,
- Laser generates the spectra and
- The source generates the light created in those spectra.

The spectra thus generated were printed on the attached printer and spectral analysis was performed by comparison of peaks their shapes and heights created at different wavelength by the FT-IR. Spectra of pure distilled water alone were also used to generate spectra of background.

RESULTS

Comparison of Spectra of Whole cells between B: 6 AND B: 3, 4

When these strains B: 6 and B: 3, 4 whole cells were subject to FT-IR analysis, differences were present for both strains. B: 3, 4 whole cells

showed major peak at 644 wave number (cm-1) and absorbance at 5.90, while B: 6 whole cells showed major peak at 628, 640 and 651 wave number (cm-1) and at absorbance of 0.35nm,0.66 and 0.67 respectively.

Comparison of Spectra of Envelope between B: 6 and B: 3, 4

When envelope preparations were subject to FT-IR analysis, again differences were present in both strains. The spectral peak was found in B: 3, 4 envelope at 646 wave number (cm-1) and at absorbance 0.75. The spectral peaks were found in B: 6 envelops at 628, 640 and 651 wave number (cm-1) and at absorbance of 0.35.0.43 and 0.24 respectively, confirming the differences of both the envelop preparation in FT-IR analysis.

Comparison of Spectra of OMPS between B: 6 and B: 3, 4

When OMPs preparations of B: 6 and B: 3, 4 OMPs was subjected to FT-IR analysis, similar results as of envelop were reviewed, indicating differences in spectral peaks in two strains, spectral peak was found in B: 3, 4 OMPs at 646 wave number (cm-1) and absorbance 5.90, while spectral peak was found in B: 6 OMPs at 628, 640 and 651 wave number (cm-1) and absorbance at 0.60, 0.50 and 0.21 respectively.

Comparison of Individual Serotypes of Whole Cells, Envelope and OMPS B: 6, & B: 3, 4

The FT-IR spectra of *P. multocida* strain (B: 3, 4) whole-cell, envelope and OMPs were observed. In these few differences were observed but more similarity were found in the spectra of the strain (B: 3, 4). Spectral peak were found in B: 3, 4 between 640-660 wave number (cm-1) and absorbance between 0.01-6. The major spectral peak was found in B: 3, 4 whole cells at 644 wave number (cm-1) and absorbance at 5.90, envelop at 646 wave number (cm-1) and absorbance 0.75 and spectral peak was present in B: 3, 4 OMPs at 646 wave number (cm-1) and absorbance 5.90.

The FT-IR spectra of *P. multocida* strain (B: 6) which consisted whole-cell, envelope and OMPs were observed with few dissimilarity and most of the similarities, which showed that strains give similar spectra for whole cells, envelops and OMPs. Two large spectral peaks and one small peak were found in B: 6 between 620-660 wave number (cm-1) and absorbance between 0.1-0.70. In whole cells peaks were at 628, 640 and 651 wave number (cm-1) and at absorbance of 0.35, 0.66 and 0.67 respectively. In envelops these peaks at 628, 640 and 651 wave number (cm-1) and at absorbance of 0.35,0.43 respectively and in OMPs the peaks were also at 628, 640 and 651 wave number (cm-1) and at absorbance of 0.60, 0.5 and 0.21 respectively.

Comparison between B: 6 and B: 3, 4 result showed that differences were present in B: 6 and B: 3, 4 serotypes spectra generated from preparations of whole cell, envelops and OMPs. Concluding that any form either whole cell, envelop and OMPs give rise to same results. Therefore one may use whole cell for generating spectra rather than time consuming, counter-some and difficult procedures of envelops and OMPs preparations.

DISCUSSION

Pasteurella multocida is an aerobic, chemoorganotrophic organism. Two *P. multocida* strains used in the study came from different sources. Both the strains namely B:6 from cattle and B:3,4 from deer were vaccinal strains (B: 6 for cattle for the killed injectable vaccine and B:3,4 from deer for the live intranasal vaccine). Similar to wild strains, vaccinal strains are also known for their capability of killing adult mice within 24 hours upon injections of live organisms. This study also establishes the earlier finding, where the live virulent as well as vaccinal strains killed mice in the same manner (5).

When these strains were subjected to FT-IR analysis of spectral peaks were found within the preparations of whole cells, envelope and OMPs of each strain shown in Figure of each. The spectra of pure distilled water alone were also used to generate spectra of background.

Comparison of Spectra of Whole Cell between B: 6 and B: 3, 4

When these strains B: 6 and B: 3, 4 whole cells were subject to FT-IR analysis of spectral peaks

were found, differences were present in both strains, B: 3, 4 whole cells showed major peak at 644 wave number (cm-1) and absorbance at 5.90 while B: 6 whole cells showed major peaks at 628, 640 and 651 wave number (cm-1) and at absorbance of 0.35nm, 0.66 and 0.67 respectively. The data indicates that both the strains had structural differences at molecular level which were shown by FT-IR spectra when solution contained whole cells in distilled water.

Comparison of Spectra of Envelops of B: 6 and B: 3, 4

When these strains B: 6 and B: 3, 4 envelops were subjected to FT-IR analysis of spectral peaks were found, difference were present in both strains. The spectral peak was found in B: 3, 4 envelop at 646 wave number (cm-1) and absorbance 0.75nm and spectral peaks were found in B: 6 envelops at 628, 640 and 651 wave number (cm-1) and at absorbance of 0.35.0.43 and 0.24 respectively confirming the differences of both the envelop preparation in FT-IR analysis.

Comparison of Spectra of OMPS between B: 6 and B: 3, 4

When these strains B: 6 and B: 3, 4 OMPs were subject to FT-IR analysis of spectral peaks were found, differences were present in both strains. Spectral peak was found in B: 3, 4 outer membrane protein at 646 wave number (cm-1) and absorbance at 5.90, while spectral peak was found in B: 6 OMPs at 628, 640 and 651 wave number (cm-1) and absorbance at 0.60, 0.50 and 0.21 respectively. These results showed the difference in spectra of OMPs preparation in FT-IR analysis.

Comparison of Spectra Preparations whole cells, envelope and OMPS among B: 6 and B: 3, 4

The FT-IR spectra of *P. multocida* strain (B: 3, 4) whole-cell, envelope and OMPs were observed. Differences in OD of the relevant spectral peaks showed the differences in the quantity of bacterial components in the samples of same serotype. In these strains, few differences were present but more similarities were found in the spectra between the preparations of same strain. In B:3,4 spectral peak were found between 640-660 wave number (cm-1) and at absorbance between 0.01-6. In whole cells the major spectral peak was found at 644 wave number (cm-1) and at absorbance at 5.90, envelope at 646 wave number (cm-1) and at absorbance 0.75; and in OMPs spectral peak was present at 646 wave number (cm-1) and at absorbance 5.90.

The FT-IR spectra of *P. multocida* strain (B: 6) preparations consisted whole-cell, envelope and OMPs were observed with few dissimilarity but at most place, the similarities were present, which shows that B: 6 gave similar spectra for whole cells, envelope and OMPs. Two large spectral peaks and one small peak were found in B: 6 all preparations fall between 620-660 wave number (cm-1) and at absorbance between 0.1-0.70. In whole cells peaks were at 628, 640 and 651 wave number (cm-1) and at absorbance of 0.35, 0.66 and 0.67 respectively. In envelope these peaks were at 628, 640 and 651 wave number (cm-1) and at absorbance of 0.35, 0.43 and 0.24 respectively and in OMPs the peaks were also at 628, 640 and 651 wave number (cm-1) and at absorbance of 0.60, 0.5 and 0.21 respectively. Differences in OD of the relevant spectral peaks show the differences in the quantity of bacterial components in different bacterial samples.

This may constitute the first report of its kind on *P. multocida*, since there appears no published work on the vaccinal strains of *P. multocida* using FT-IR. The comparison of spectra of individual strain preparations of whole cell, envelope and OMPs shows similarity. Therefore one can use any preparation of strain whole cells, envelope and OMPs. Therefore one may use whole cells for generating spectra rather than time consuming, cumbersome and difficult procedures of envelope and OMPs preparations.

During present study attempt was made to highlight the extent of similarities and dissimilarities among the strains of *P. multocida*. A detailed study with diversified parameters need to be under taken with larger number of *P. multocida* strains / isolates /serotypes to gain more insight into the structural and functional relationship and epidemiology of the diseases

ultimately leading to the better control of the this most important economic disease of cattle in the country.

REFERENCES

1. Carter, G.R. (1955). Studies on *Pasteurella multocida* I. A haemagglutination test for the identification of serological types, Am. J. Vet Res., 16, 481–484.
2. Carter, G.R.; De Alwis M.C.L. (1989). Haemorrhagic Septicemia. In: Pasteurella and Pasteurellosis, (Academic Press Limited, London).
3. Chawak, M.M.; Verma, K.C.; Kataria, J.M. (2001). Characterization and immunization properties of outer membrane proteins of *Pasteurella multocida* grown in iron sufficient and restricted media. Indian Journal of Comparative Microbiology, Immunology and Infectious diseases, 21, 36- 42.
4. De Alwis M.C.L. (1992). Pasteurellosis in Production Animals: A Review. In: Pasteurellosis in Production Animals, (ACIAR, Bali, Indonesia).
5. De Alwis, M.C.L. (1999). Hamemorrhagic septicemia. Australian Center for International Agricultural Research, 57,141.
6. Helm, D.; Labischinske, H.; Schallehnn, G.; Naumann, D. (1991). Classification and identification of bacteria by Fourier transform infrared spectroscopy. J .Gen. Microbiol, 137, 69-79.
7. Kim, S.; Reuhs, B.L.; Mauer, L.J. (2005). Use of Fourier transform infrared spectra of crude bacterial Lipopolysaccharides and chemometrics for differentiation of Salmonella enterica serotypes. J. Appl. Microbiol, 99,411-417.
8. Knights, J.M.; Adlam, C.; Owen, P.L. (1990). Characterization of envelop proteins from Pasteurella haemolytica and *Pasteurella multocida*, J. Gen. Microbiol, 136, 495-505.
9. Lin, J.S.; Huang.; Zhang, Q. (2002). Outer membrane proteins: key players for bacterial adaptation in host niches, Microbiol. Infect, 4,325–331.
10. Muniandy, N.J.; Edgar, J.B.; Woolcock.; Mukkur, T.K.S. (1993). Virulence, purification, structure, and protective potential of putative capsular polysaccharide of *Pasteurella multocida* type B:6 (Pasteurellosis in Production Animals).
11. Namioka, S.; Bruner, D.W. (1963). Serological studies on *Pasteurella multocida*. IV. Type distribution of the organisms on the basis of their capsule and O groups. Cornell Vet, 53, 41-53.
12. Naumann, D.; Keller, S.; Helm, D.; Schultz, C.; Schrader, B. (1995). FT-IR spectroscopy and FT-Raman spectroscopy are powerful analytical tools for the non-invasive characterization of intact microbial cells. J. Mol. Struct, 347, 399–406.Stuart, B. (2004). (Chichesture UK Valley).
13. Prince, G.H.; Smith, J.E. (1996). Ultracentrifugation as a means for the separation and identification of LPS. ACS symposium series, 419,238-249.
14. Rimler, R.B. (1990). Comparisons of *Pasteurella multocida* Lipopolysaccharides by sodium dodecy1 sulfate- polyacrylamide gel electrophoresis to determine relationship between group B and E hemorrhagic septicemia strains and serologically related group A strains. J. Clin. Microbiol, 28,654-659.

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FIGURES

Fig1. B: 3, 4 Whole Cell

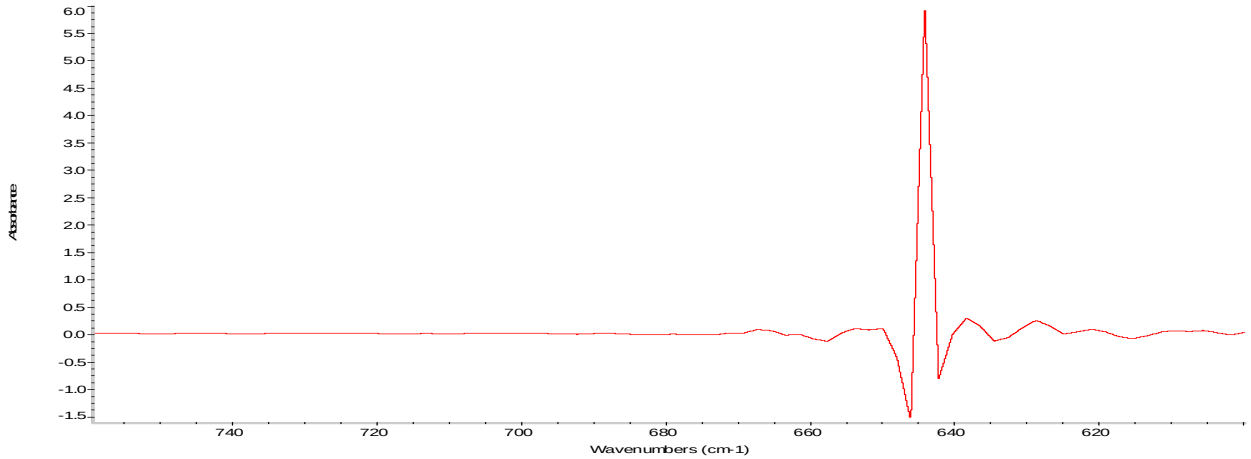

Fig 2. B: 6 Whole Cells

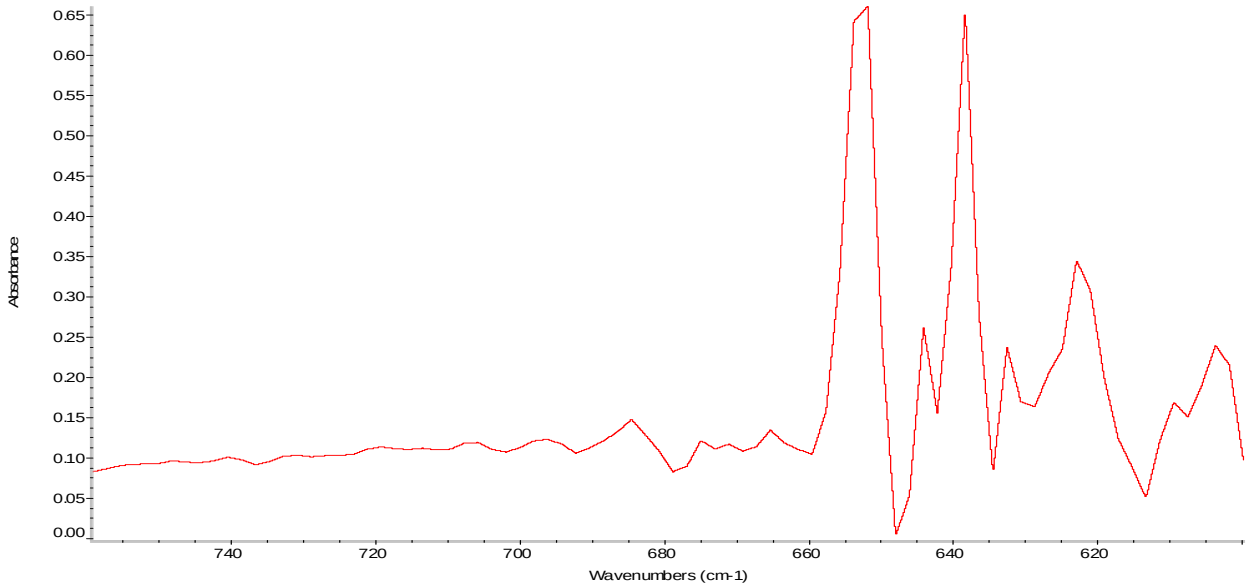

Fig 3. B: 3, 4 Envelope

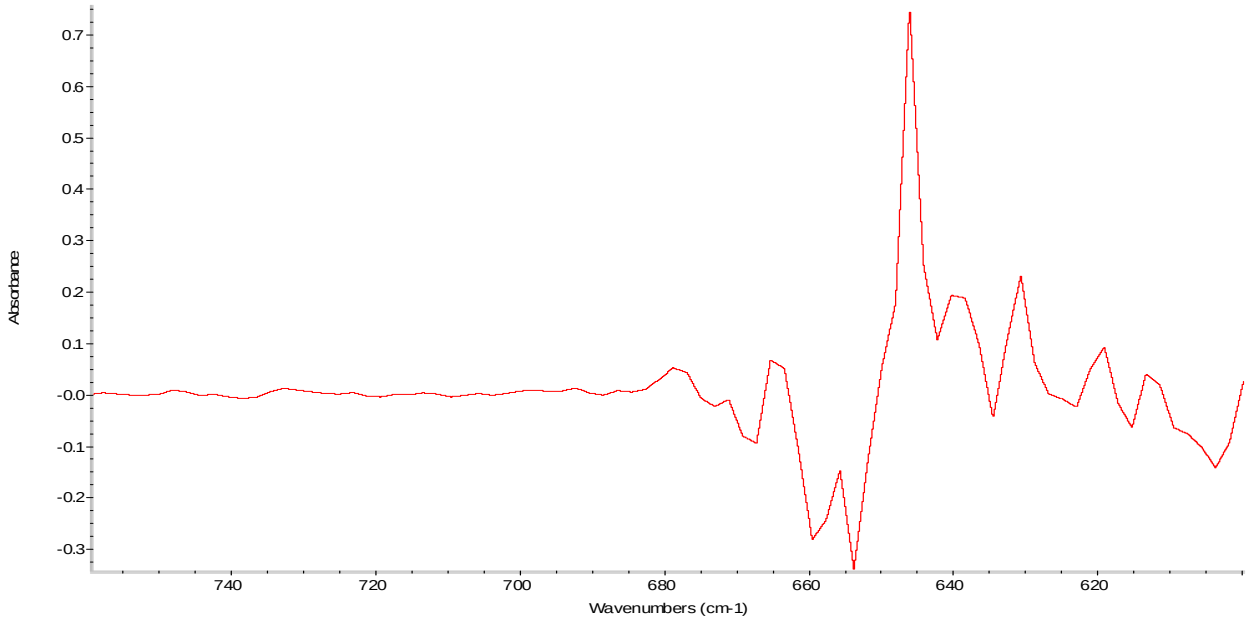

Fig 4. B: 6 Envelope

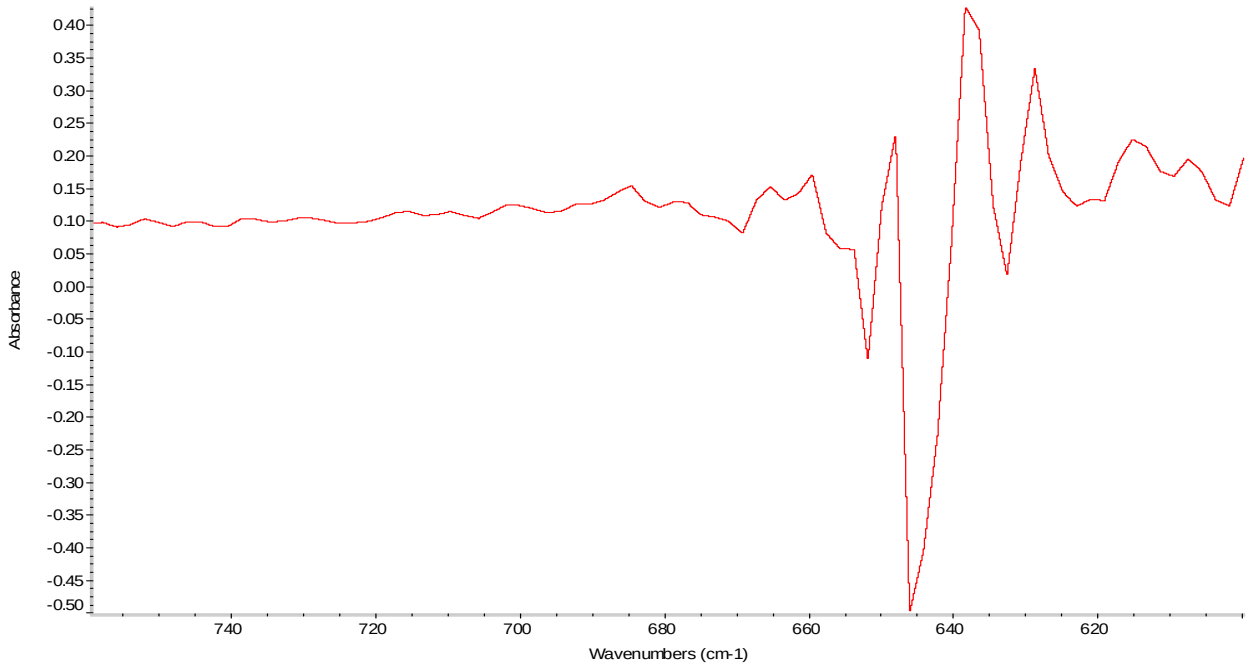

Fig 5. B: 6 Outer membrane proteins

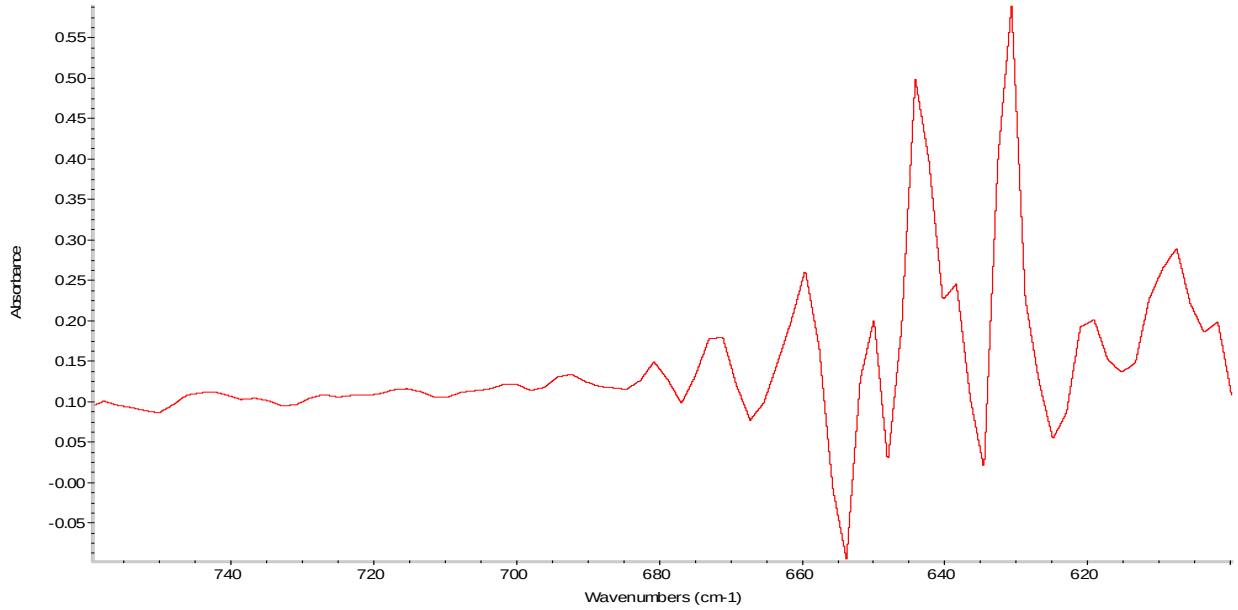

Fig 6. B: 3, 4 Outer membrane proteins