



Original Research

Effect of Iron Restriction on the Whole Cell and Outer Membrane Proteins of *Riemerella anatipestifer* Isolates of Kerala

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Abstract

Globally, *riemerellosis* is caused by *Riemerella anatipestifer*, has a major concern, especially around the duck producing countries in variable degrees. A contemplate was led to access the homogeneity/heterogeneity between the *Riemerella* isolates (RA1 and RA2) by comparing the whole cell and outer membrane protein profiles in iron restricted and repleted condition. On Sodium dodecyl sulphate polyacrylamide gel electrophoretic analysis, both the isolates under iron deprived condition, commonly increased the production of outer membrane proteins, 51 and 75 kDa, among the five increased proteins in each. Also, among the increasingly produced eight and six whole cell proteins of RA1 and RA2, respectively, 38 and 51 kDa were present in both under iron depleted condition. The present study showed heterogeneity in the protein profile of *Riemerella* isolates, RA1 and RA2. Both the isolates, under iron restricted conditions, not only up and down regulate various whole cell and Omp proteins, but also few novel proteins had been produced in iron depleted environment.

Key words: Ducks, Kerala, New Duck Disease, Outer Membrane Protein Profiles, *Riemerellosis*, *Riemerella Anatipestifer*, Whole Cell

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Introduction

Riemerella anatipestifer (RA) is a Gram-negative, non-spore forming and non-motile bacilli. This etiological agent leads to new duck disease or *riemerellosis*, an infectious, enzootic, malady of young ducklings. Major and minor polypeptides analysed in the whole cell and outer membrane protein profile constitutes a noteworthy segment of immunogenic moiety of the bacterium and will be a useful tool for future vaccine development contemplates. Iron is one of fundamental supplements for the survival of the microorganisms and hosts. Under iron deprived condition, hosts withhold iron from pathogens to avoid





bacterial development, while bacteria up and down control the production of numerous proteins amid iron depletion and may deliver new immunogenic proteins (Salysers and Whitt, 2002). Though the whole genome of *R. anatipestifer* has been sequenced and immunoproteomics were reported, the gene expressions are not well known during iron deficiency condition till now. Considering all these realities, present study was concerned with the objectives of analyzing the expression of whole cell and outer membrane proteins of *R. anatipestifer* isolates grown under iron-restricted and iron-repleted conditions.

Materials and Methods

Revival of Bacteria

Among the different isolates of *R. anatipestifer*, one each of predominant (RA1) and lesser predominant (RA2) isolates maintained in the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy were revived and identified.

Growth under Iron Sufficient and Deficient Conditions

R. anatipestifer isolates (RA1 and RA2) were cultured in tryptic soy broth (TSB) at 37°C. At the exponential growth phase ($OD_{630nm} \sim 0.5$), bacterial culture was divided into two tubes, each having 5 mL TSB medium. Added 200 μ M of 2, 2'-dipyridyl into one of the tube in order to remove iron from the medium whereas the other tube kept free of addition. These two cultures were placed in the shaker incubator (220 rpm) at 37°C for 16 hrs.

Extraction of Proteins

The whole cell and outer membrane protein extractions were performed as per the method described by Srivastava (1998) and Davies and Donachie (1996), respectively.

Whole Cell and Outer Membrane Protein Profiling

Analysis of whole cell and outer membrane protein of *R. anatipestifer* was carried out by employing one-dimensional SDS - PAGE as per the method described by Laemmli (1970) with relevant modifications.

Results and Discussion

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is one of such an efficient technique to verify and analysed different serovars of *Riemerella* from the extracted cell lysates. It even helpful to identify the immunogenic whole cell and outer membrane protein segments of the bacterium and will be a vital tool for vaccine development contemplates in future. During unfavorable conditions, some bacteria follows the Darwinian evolutionary theoretic phrase, "Survival of the fittest", for their existence by changing in the pattern of protein production with respect to the varying gene expression. Under iron repleted and iron depleted environment, bacterial status is variable in the production rate of proteins,



number of immunogenic proteins and production of novel proteins. It is the thrilling experience to explore through unseen miraculous microbial world. So, the present study was to estimate the range of similarity between the isolates of *R. anatipestifer* by SDS-PAGE of whole cell and outer membrane proteins under iron enriched and iron depleted environment.

Revival and Identification of *R. anatipestifer*

Both the isolates were revived successfully. The results of cultural characteristics, morphology and biochemical tests were in accordance with the earlier workers (Vancanneyt *et al.*, 1999; Bernardet *et al.*, 2002; Shome *et al.*, 2004; OIE, 2010, Sabnam, 2015; Surya *et al.*, 2016). The only difference was noted in the urease test, that RA1 was positive but RA2 was negative, which was in accordance with Shancy (2015). The *R. anatipestifer* species- specific PCR revealed an amplicon at 546 bp as like the findings of Soman *et al.* (2014) and Shancy (2015).

Estimation of Whole Cell and Outer Membrane Proteins

The concentration of whole cell and outer membrane proteins of *R. anatipestifer* isolates grown under varied atmosphere were performed by Lowry's method. The concentration of whole cell protein of RA1 and RA2 was 1.57 mg/ml and 1.49 mg/ml, respectively and the Omp concentration of RA1 and RA2 was 490 µg/ml and 450 µg/ml, respectively in iron enriched conditions. Under iron restricted conditions, the concentration of whole cell protein of RA1 and RA2 was 1.41 mg/ml and 1.24 mg/ml, respectively and the Omp concentration of RA1 and RA2 was 475 µg/ml and 435 µg/ml, respectively. The estimated Omp concentration of *R. anatipestifer* was 1.31 mg/mL by Ahmad (2017). As per Shancy (2015), the Omp concentration of RA1 and RA2 was found to be 1.32 mg/mL and 1.35 mg/mL.

Protein Profiling of RA1 and RA2 *Riemerella* Isolates

The standard protein marker ranges from 10 kDa to 250 kDa was used in the study. Between RA1 and RA2, there is approximate 50 per cent similarity in accordance with their outer membrane protein profile (Fig. 2 and Fig. 3). But in accordance with their whole cell protein profile, approximate 56 per cent of similarity has been shown (Fig.1- Lane A and C). The dissimilarity in the protein profile of both isolates revealed their heterogeneity. As per Segers *et al.* (1993) among the strains of *R. anatipestifer*, more than 85 per cent parallelism was observed in the profiles of whole cell protein by SDS-PAGE. Longfei *et al.* (2005) observed that the molecular weight of ten proteins were similar between their local isolates (RA1 and RA2) out of 13 Omp, with molecular weight ranging from 20 to 100 kDa.

Whole Cell and Membrane Protein Profiles of RA1 and RA2

Between 21 whole cell and 20 outer membrane proteins of RA1, 93 per cent similarity has been observed whereas 76 per cent similarity has been shown between the 18 whole cell and 16 outer membrane proteins of RA2 in accordance to the molecular weight of proteins (Fig. 1, 2 & 3).

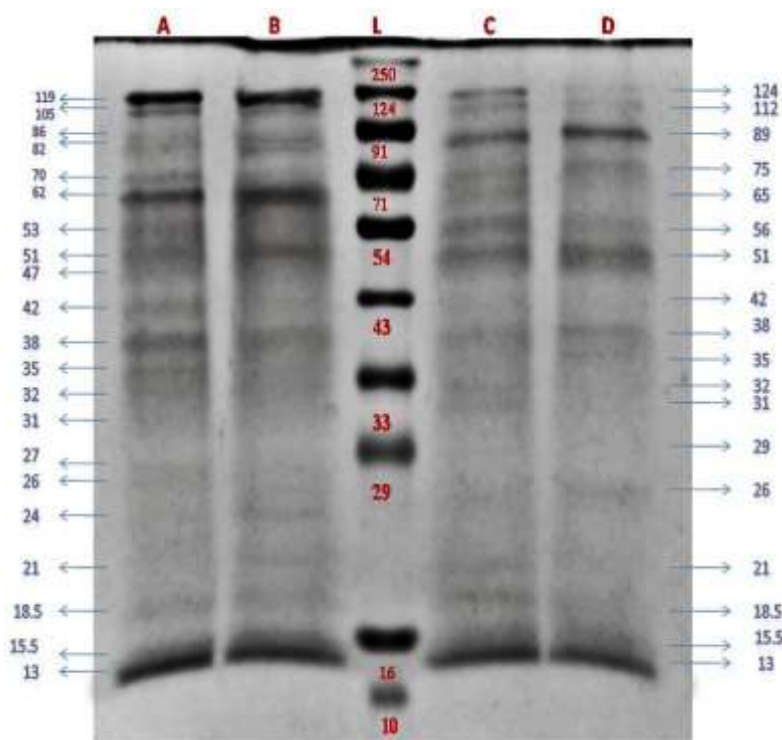


Fig 1: SDS-PAGE of the whole cell proteins of RA1 and RA2 under iron enriched and iron restricted conditions (**A**- Whole cell proteins of RA1; **B**-Iron restricted whole cell proteins of RA1; **L**- Protein ladder; **C**- Whole cell proteins of RA2; **D**- Iron restricted whole cell proteins of RA2)

The difference in the whole cell protein has been observed by the presence of 26 and 27 kDa protein bands which were absent in the outer membrane. Instead, 75 kDa was present in the outer membrane which was absent in the whole cell proteins of RA1. Common bands present in the whole cell and outer membrane protein profiles of RA1 were 119, 105, 86, 82, 70, 62, 53, 51, 47, 42, 38, 35, 32, 31, 24, 21, 18.5, 15.5 and 13 kDa. In RA2, Omp was unique with the presence of 250, 190 and a light 24 kDa protein band but whole cell had 112, 42, 38, 29 and 13 kDa unique bands which were absent in outer membrane protein. Common bands present in the whole cell and outer membrane protein profiles of RA2 were 15.5, 18.5, 21, 26, 31, 32, 35, 51, 56, 65, 75, 89 and 124 kDa.

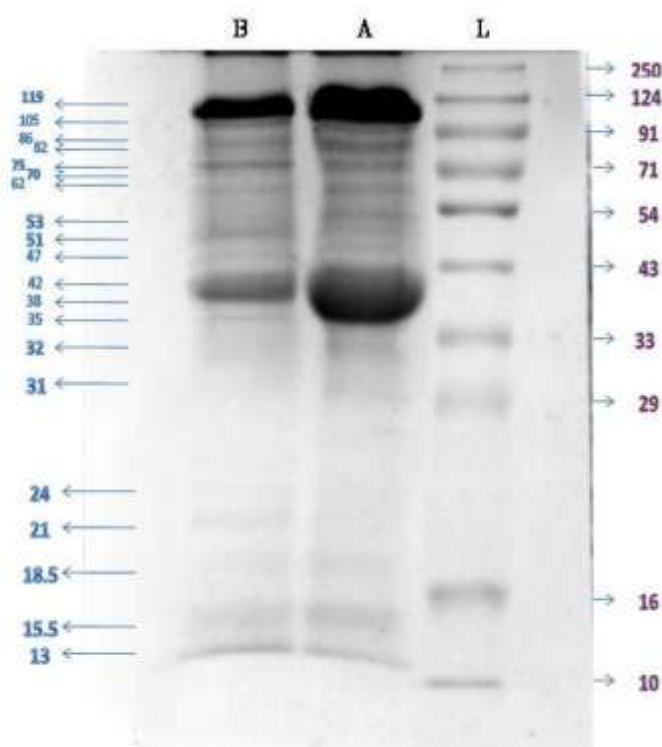


Fig. 2: SDS-PAGE of the outer membrane proteins of RA1 under iron enriched and iron restricted conditions (L- Protein ladder; A-Omp of RA1; B-Iron restricted Omp of RA1;)

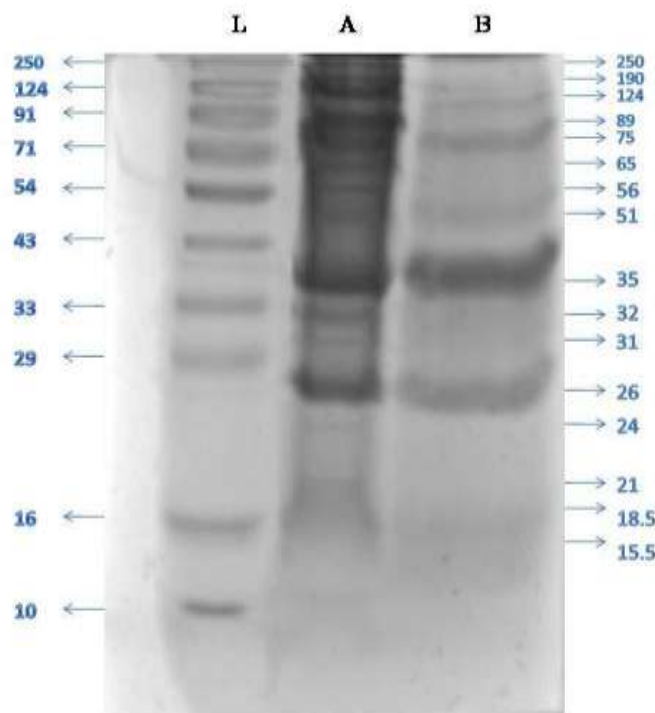


Fig. 3: SDS-PAGE of the outer membrane proteins of RA2 under iron enriched and iron restricted conditions (L- Protein ladder; A- Omp of RA2; B- Iron restricted Omp of RA2)



Sulochana (1998) noticed four unique protein bands of 34, 33, 26.9 and 4 kDa in *P. multocida* which was absent in *R. anatipestifer* in iron sufficient conditions, 10 bands were common to both and three bands of 43, 23 and 10.5 kDa were unique to *R. anatipestifer* on SDS-PAGE analysis. Somu (2014) found several protein bands like 40, 41, 42, 60, 62, 68, 70, 190 and 205 kDa and Ahmad (2017) observed 30, 35, 42 and 60 kDa of *R. anatipestifer* protein bands in the profile of Omp sufficient Sarkosyl insoluble region on SDS-PAGE under iron sufficient condition.

Most of the PAGE protein bands of *R. anatipestifer* lysates were normal to all serotypes whereas few were constrained to a group of serotypes or a solitary serotype (Higgins *et al.*, 2000). Under iron sufficient condition, Surya (2011) revealed 14 to 15 visible bands of whole cell proteins from the six *R. anatipestifer* isolates (RA1 to RA6) with molecular weight from 93 kDa to 28 kDa.

Profile of the Whole Cell and Membrane Proteins of *R. anatipestifer* Isolates Grown under Iron Repleted and Iron Depleted Conditions

The growth condition of *R. anatipestifer* under iron enriched and iron depleted conditions increased the production of some proteins and at the same time, decreased the production of some other proteins due to up and down regulation of certain genes and their further expression of proteins in choice of the living environment. Out of 21 whole cell proteins, six of them had increased production like 15.5, 38, 51, 62, 82 and 86 kDa and slight increase in the production of 21 and 24 kDa whereas out of 20 outer membrane proteins, 38, 51 and 75 kDa proteins had shown increased production much more than that of 21 and 24 kDa in iron restricted condition of RA1. The whole cell protein bands like 38, 51, 75 and 89 kDa had produced much more than 26 and 35 kDa among the increased proteins and among the outer membrane proteins, 26, 35, 51 and 75 had increased tremendously and slight increase was observed to 124 kDa protein, under iron restricted condition of RA2. Remaining protein bands in both RA1 and RA2 had decreased production.

As per Yang *et al.* (2013) 23 proteins were drastically increased and three novel proteins were found under iron deprived media of *R. anatipestifer*, Serotype 1 using 2-D PAGE. Riean_1750 and Riean_1752 were the two novel insoluble proteins having molecular weights, 34 kDa and 23 kDa, respectively. Liu *et al.* (2017) revealed that many genes were distinguishably down regulated under iron deprived condition compared to the count of upregulated genes of *R. anatipestifer* by analyzing the transcriptomic conditions.

Conclusion

According to the results of present study, heterogeneity exists between the two isolates of *R. anatipestifer*, RA1 and RA2 by estimating the whole cell and outer membrane protein profiles. Both the isolates, under iron restricted conditions, not only up and down regulate various whole cell and Omp proteins, but also few novel proteins had been produced. To develop potent vaccines in future, efficacious immunogenic proteins could be identified by immunoblotting.



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