Introduction to Proteomics
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Lecture - 14
DE Applications (contd) & Challenges

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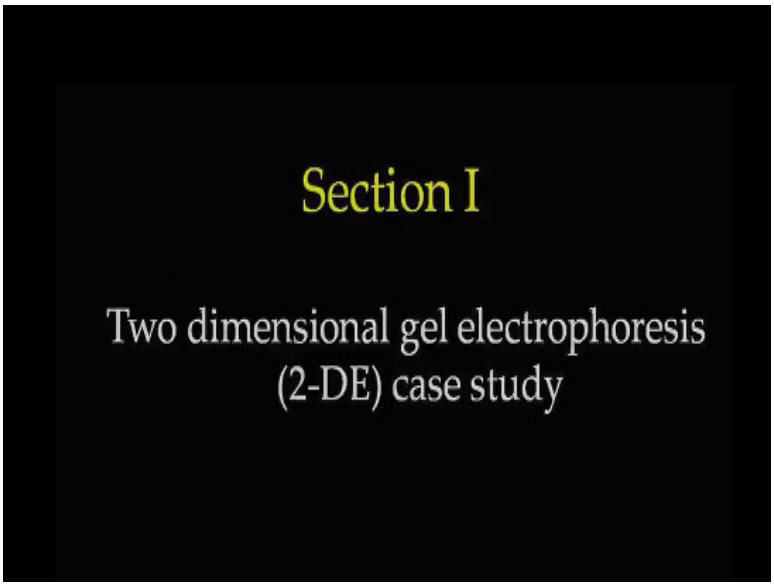

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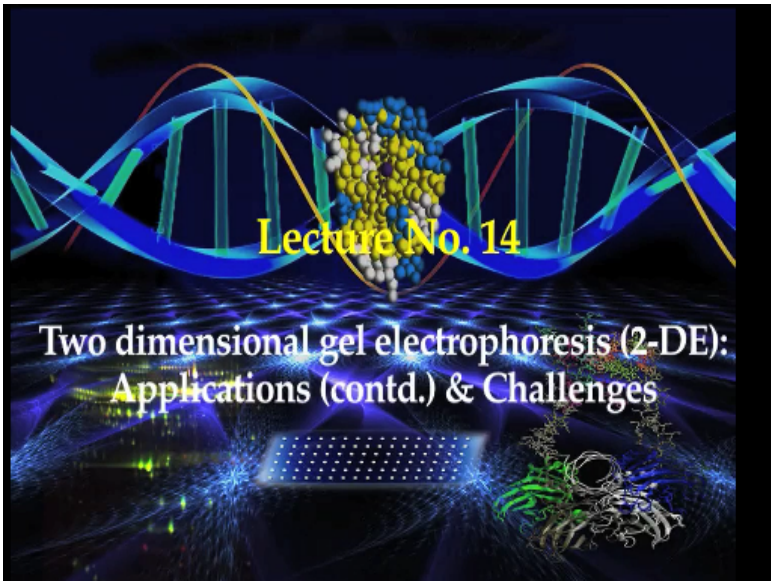

In today's lecture, we will talk about-Applications of two-dimensional electrophoresis if you recall in the previous picture we started discussing about some case studies about two

dimensional forces applications. So, as you have studied these modules what are different type of workflow involved in performing two-dimensional flow experiments. We have gone through step by step starting from the protein extraction doing the quantification.

Isoelectric focusing which separates protein in their first dimension followed by doing the equilibration repairing these strips for second dimension separation. And then you separate the proteins on the SDS page based on the molecular weight followed by it stain the gels to see the visualize the spot and then scan those images analyze that by using various software and then perform some statistical analysis to obtain some biological insight.

Now, this workflow remains the same regardless of whatever application one wants to use in their different type of biological questions. I started discussing about two-dimensional electrophoresis application in last class I gave you an overview of the 2DE at that time So, let us continue our todays lecture from the same theme and let us discuss some more case studies on how people have employed two-dimensional electrophoresis.

The power of this technique to dissolve 1000 of proteins and compare those for various differential proteomic applications.

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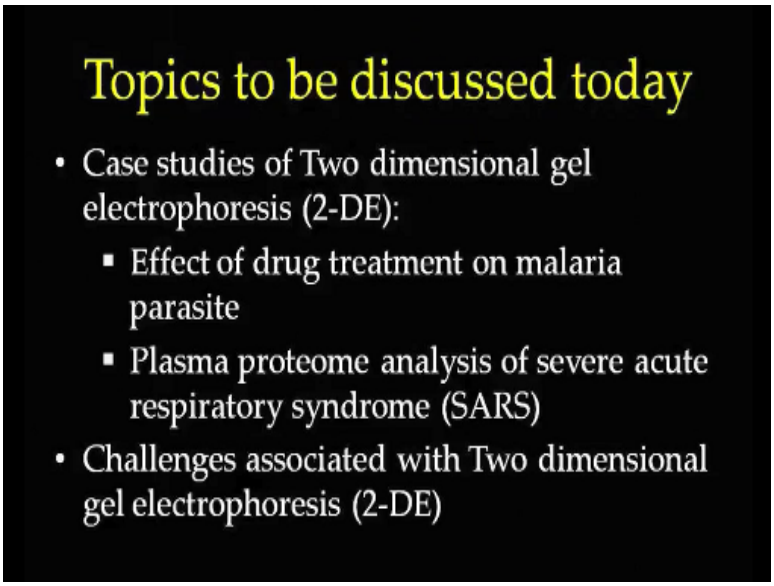

So, in today's lecture, outline first we will continue our case studies on conventional two-

dimensional electrophoresis. We will talk about a study based on the drug treatment on malaria parasite Plasmodium falciparum will then take a study on the plasma proteome analysis of SARS virus.

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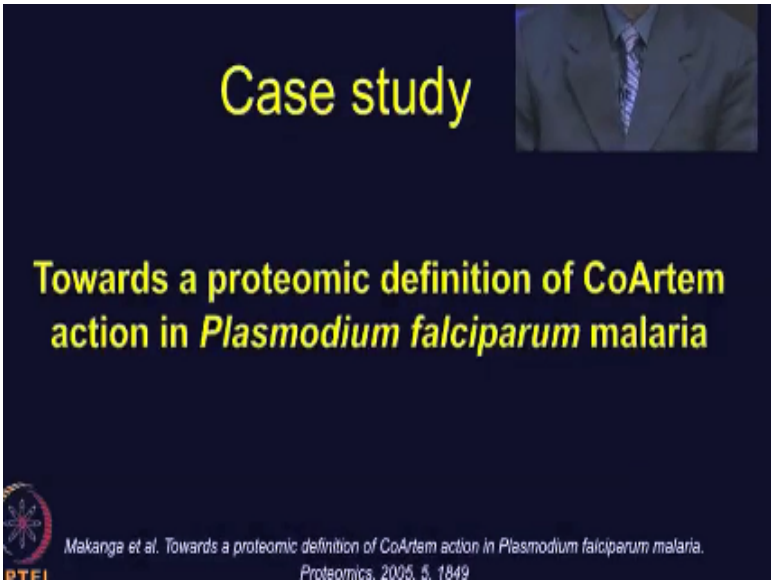

So, let us start with the first case study towards a proteomic definition of CoArtem action in plasmodium falciparum malaria. A study by Makanga et al in 2005.So, you know each year hundreds of millions of new malarias infection cases result in over 1 million deaths worldwide but due to the lack of effective vaccine and widespread resistance to the anti-malarial drugs still a lot of deaths are happening, and malaria problem is still posing challenges for its control.

The anti-malaria therapy of chloroquine and pyrithiamine did have not be able to control the mortality rate because of the anti-malarial drug resistance development. So, therefore that urgent need for identifying new drug targets as well as understanding the course of action of these drugs by applying various of high therapod techniques.

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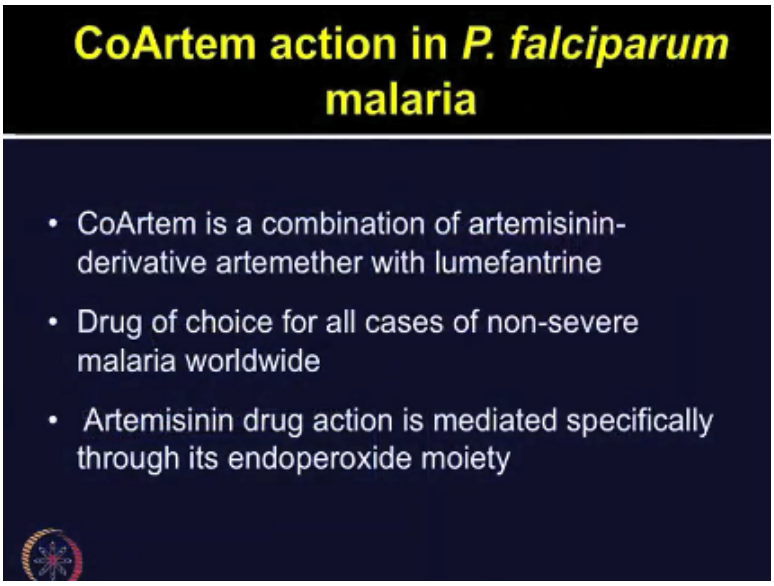

So, this paper authors have discussed how two different drugs which are effective for the antimalarials can be studied for looking at the proteome changes in the Plasmodium falciparum parasite So CoArtem is a combination of artemisinin derivative artemether with lumefantrine, how these two drugs behave and how the proteome changes occur due to the action of these two drugs were studied in this paper.

Such as applied proteomic approaches the two-dimensional electrophoresis to study the proteomic alteration of each of these drugs for these drugs that are applied as the drug of choice for all cases of non-severe malaria worldwide. The artemisinin drug action is mediated is specifically through its endoperoxide moiety. However, the more detailed mechanism of action of these drugs are still unknown.

So, the purpose of the study was to investigate the action of two active components of new anti-malarial CoArtem artemether and lumefantrine on human malaria parasite plasmodium falciparum and authors tried to look for alterations in parasite proteome which were induced by each of these drugs.

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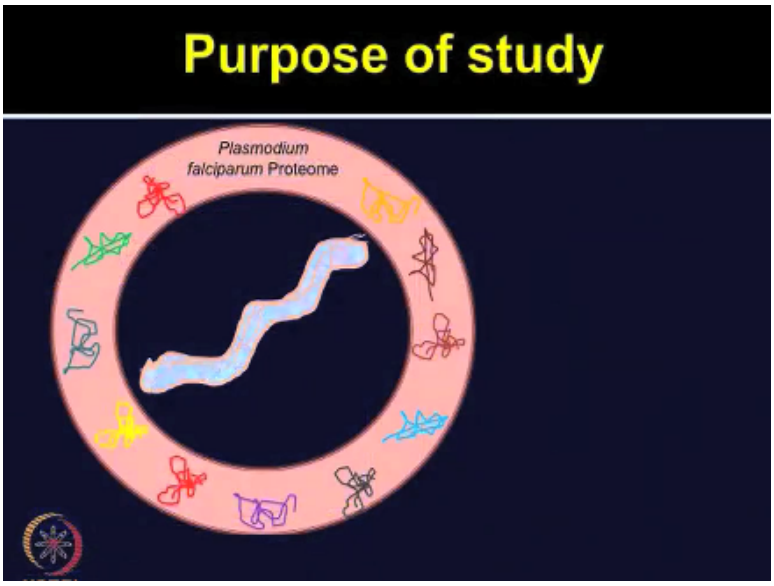

To obtain the insight of proteomic alteration they separated the proteins on the two-dimensional electrophoresis gels and compared the response of these proteomic alterations based on these two drugs. And then they identified certain proteins which were either commonly expressed due to these or they were differentially expressed due to these drugs. To certain proteins were found to be commonly upregulated due to both of these drugs and certain proteins have the different patterns.

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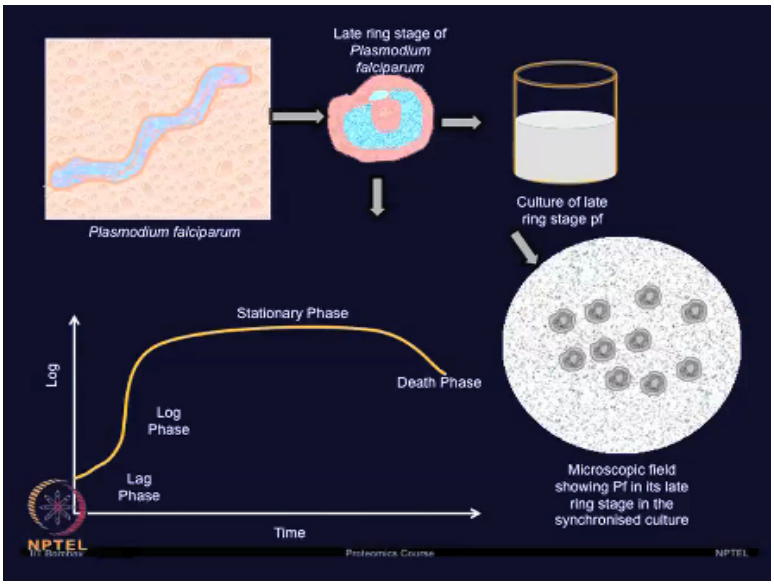

But before looking at the proteomic alterations author first determined IC10, 20, 50 and IC90 values for both the drugs ARM and LUM. We will use the abbreviations for artemisinin and lumefantrine. An effect of these concentration of drugs on parasite growth over 24 hours was

characterized as you can see the growth curve in the slide synchronized ring a stage parasite culture were harvested over 24 hours' period after the exposure to the ARM and LUM.

The parasite growth was determined by using hypoxanthine uptake ASSAY. After establishing the culture conditions and the drug concentration then authors look for the proteomic alterations So first of all they did the fractionation of the P falciparum proteome.

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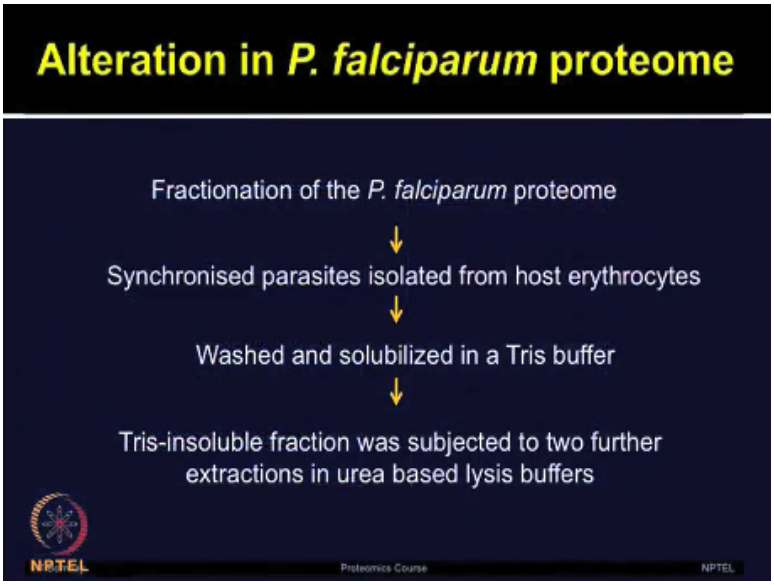

Synchronized parasites which were isolated from the host erythrocytes wash those initially and then solubilize that in a Tris buffer recipe. The Tris insoluble fraction was further subjected to extraction in the urea-based lysis buffer. As protein extraction was done then authors used IPG strip of PH 3 to 10 range for the first-dimension separation of protein in the linear IPG strips.

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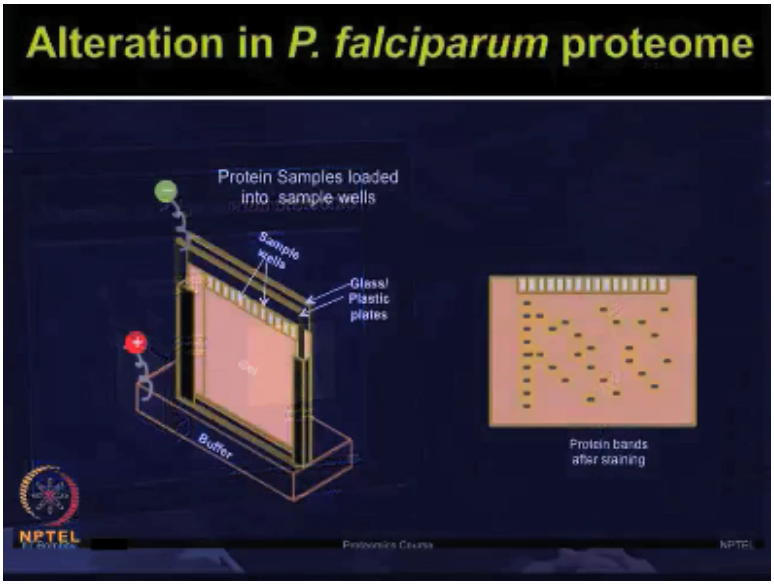

After the IF was done they equilibrated these IPG strips and then applied that on 12.5% of the vertical SDS gel. After the second-dimension separation based on the molecular weight then these gels were stained with the silver or Coomassie brilliant blue stains So, by employing two-dimensional electrophoresis and comparing the gel images by using the pedicle software Authors are able to see that there is a differential it is proteomic response which is drug specific.

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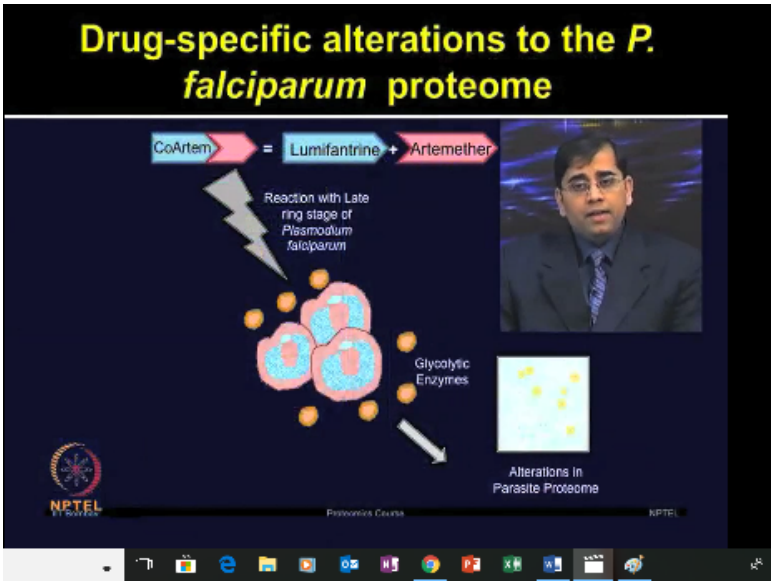

The quantitative analysis of the alternate protein expression levels following exposure to the ARM and LUM were analyzed. And then those protein spots which were differentially expressed and significant were further subjected to mass spectrometry-based analysis. So, the comparative analysis of 2d gels from untreated and drug treated parasite protein fractions provided direct and

distinct alterations in parasite proteome following artemether or lumefantrine drugs.

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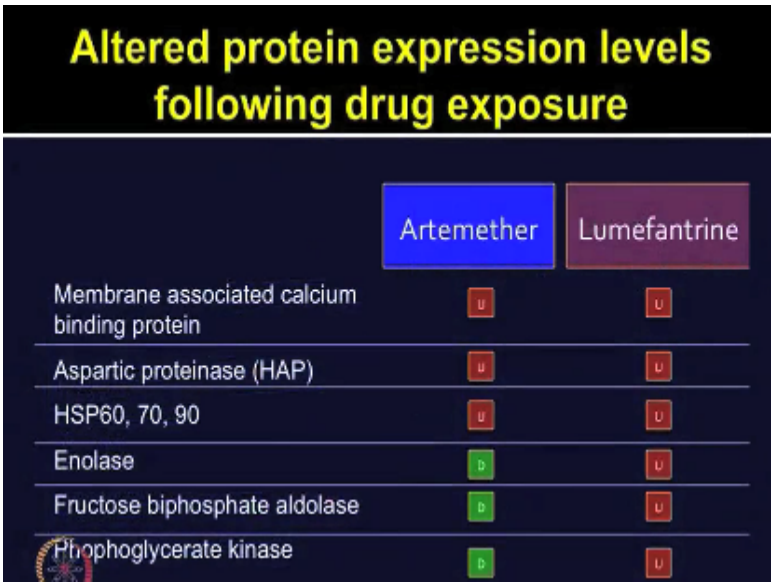

Certain proteins were identified a few of those showed a common response due to both drugs. However, there are certain proteins which showed opposite trends due to each of these drugs. Proteins such as membrane associated calcium binding protein was upregulated in both the drugs. Aspartic proteinase was also up regulated in both the cases heat shock proteins such as HSP 60,70 and 90 those were upregulated due to both the drug treatment.

There are certain proteins such as Enolase, Fructose bisphosphate aldolase and phosphoglycerate, kinase these proteins were down regulated in Artemether treatment and upregulated in the Lumefantrine treatment. So, interestingly the ARM treatment reflected in the more than 3-fold down regulation of the glycolytic enzymes such as Enolase, phosphoglycerate kinase, fructose bisphosphate aldolase and glyceride 3 phosphate dehydrogenase.

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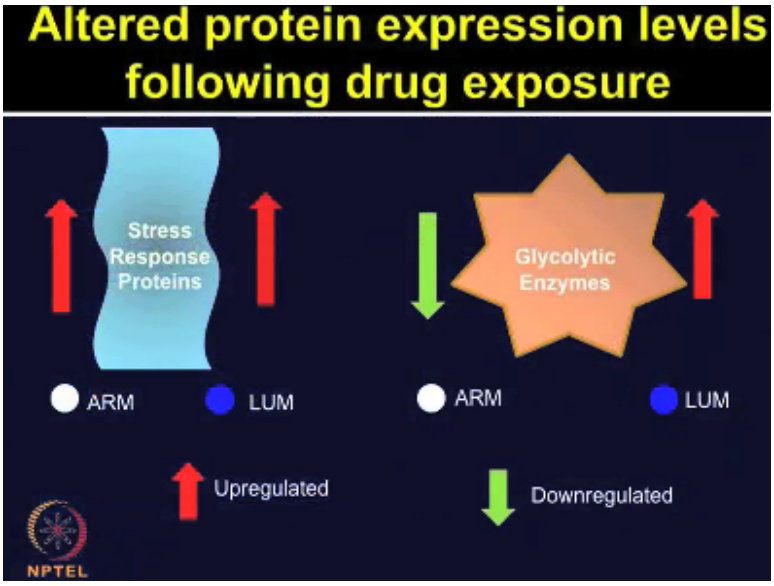

The expression of the same enzymes was also up regulated more than 3 forth due to the lumefantrine treatment. However, the certain proteins such as stress responsive proteins like heat shock proteins which were commonly induced due to either of these drug treatments which looks like a general stress response as compared to any unique response to the given drugs. So, from this study the major findings were the thought authors successfully investigated.

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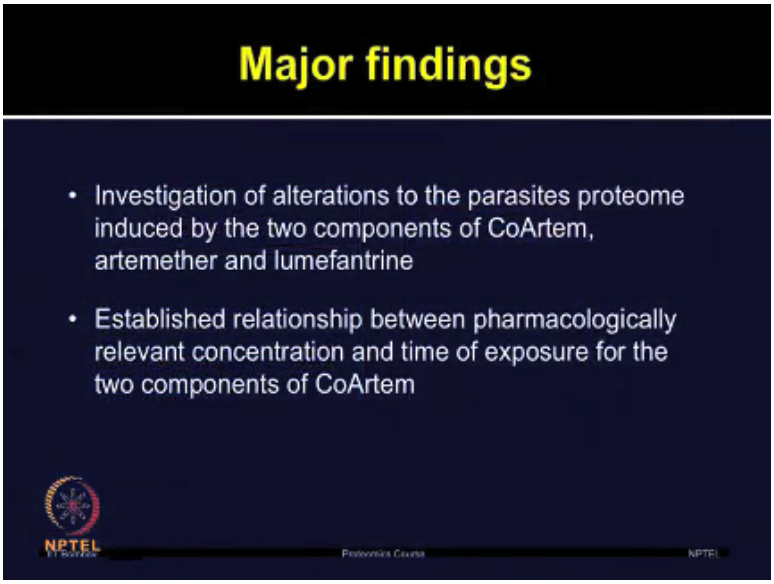

Alterations of parasites proteome induced by two components of CoArtem artemether and lumefantrine by using proteomic approach they investigated a specific and nonspecific effects of 2 anti-malarial drugs in pharmacological relevant conditions. Expression of certain proteins were quite interesting including a membrane bound calcium binding protein which was up regulated

due to the artemether and lumefantrine treatment.

The study also established a relationship between the pharmacologically relevant concentration and time of exposure for the two components of CoArtem.

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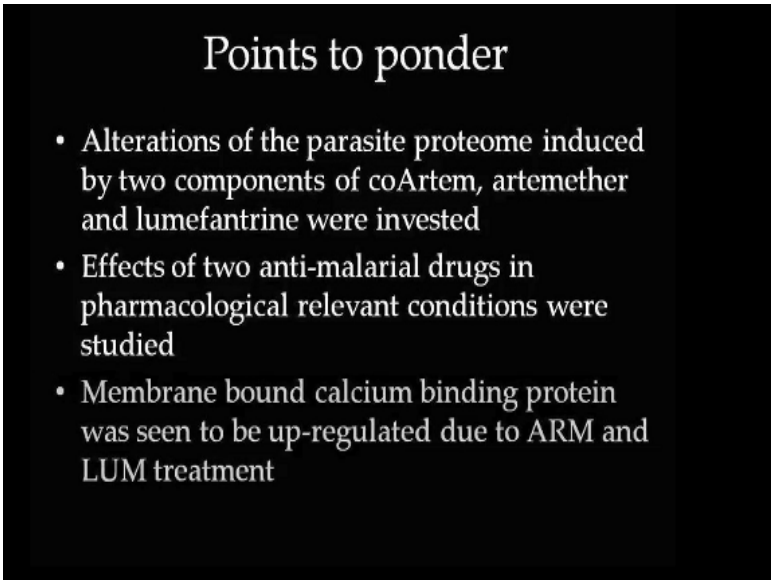

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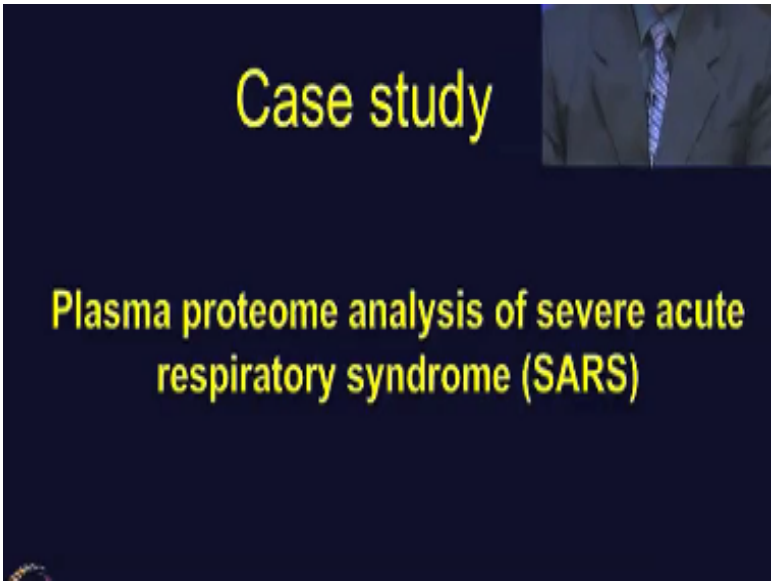

Let us now move on to case study to plasma proteome analysis of severe acute respiratory syndrome SARS a study by Chen et al in 2004. So, the purpose of the study was to perform a comprehensive plasma proteome analysis of.

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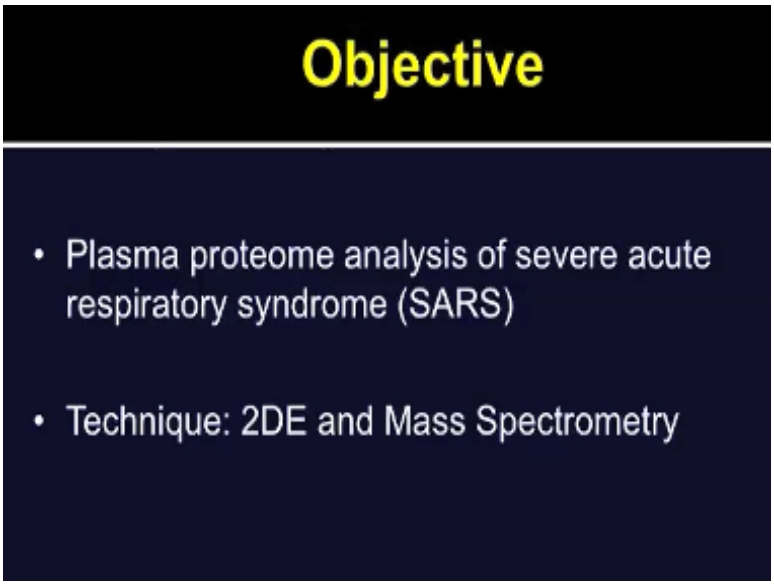

Severe acute respiratory syndrome and its comparison with the healthy individuals. In this study authors employed conventional two-dimensional electrophoresis analyzed those images from the treatment with the controls and identified the differential expressed protein by using mass spectrometry techniques.

Including multi tuft of an LC MS MS finally the interesting identified proteins were validated from techniques such as western blots. So, if you remember a few years ago the severe acute respiratory syndrome occurred in 2002-2003 and thousands of deaths were reported in several countries around the globe.

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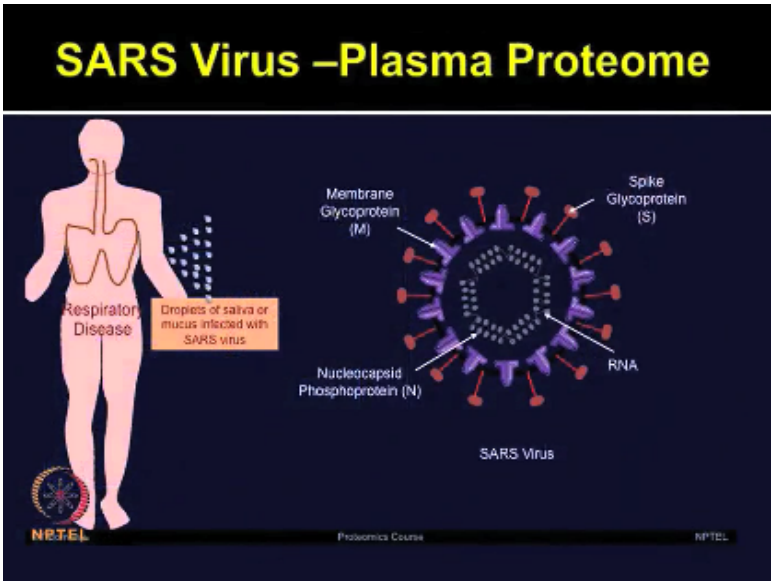

The genome sequence of SARS virus was already known and the structure of main protease and receptors were also known. However, the pathogenesis of SARS was not very clearly understood since serum or plasma they provide a very valuable sample to identify the targets for diagnostic prognostic and therapeutic. Authors use plasma sample for the analysis of proteomic alteration in the SARS patients for the plasma proteomic analysis.

Authors explore the possible pathogenetic mechanism of progression of sorts by analyzing plasma proteins of 22 different plasma samples which are obtained from the 4 SARS patient and 6 healthy controls.

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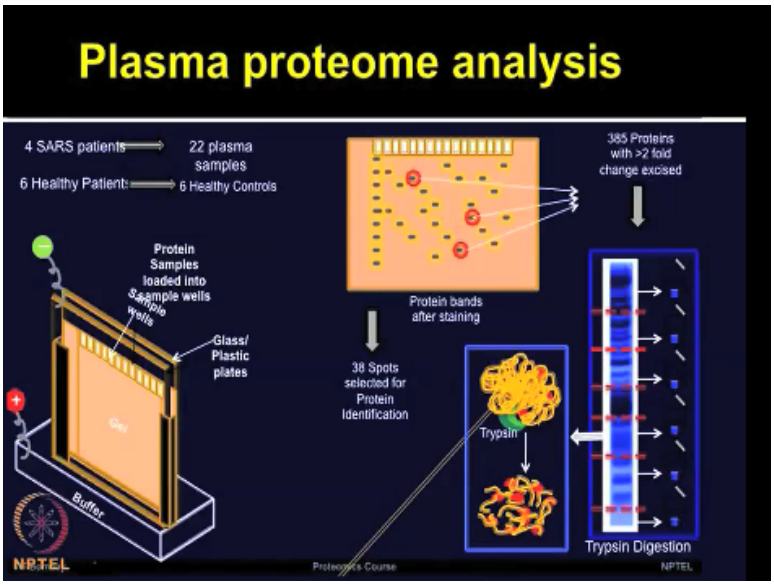

Authors analyzed plasma proteome by using two-dimensional electrophoresis on 4 to 7 PH immobilized PH gradient strips and they stained the gels with a sensitive esteem SYPRO ruby. Since the slide you can see the steps involved in the traditional two-dimensional electrophoresis followed by the mass spectrometry. Both the controls and the treatment the healthy individuals as well as patients suffering from SARS.

The plasma was separated and then proteins were resolved on the two regions differentially expressed proteins were further subjected to the in-gel Trypsin digestion followed by the mass spectrometry.

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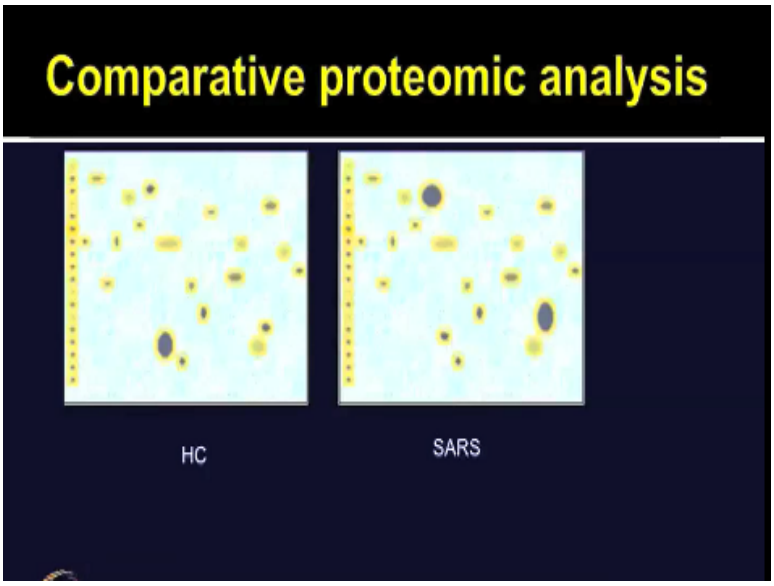

So, the completed proteomic analysis of these 2d gels revealed that 38 protein spots were differentially expressed more than 2-fold change and out of these spots there were 30 proteins which were upregulated 35 were upregulated and 3 were down regulated.

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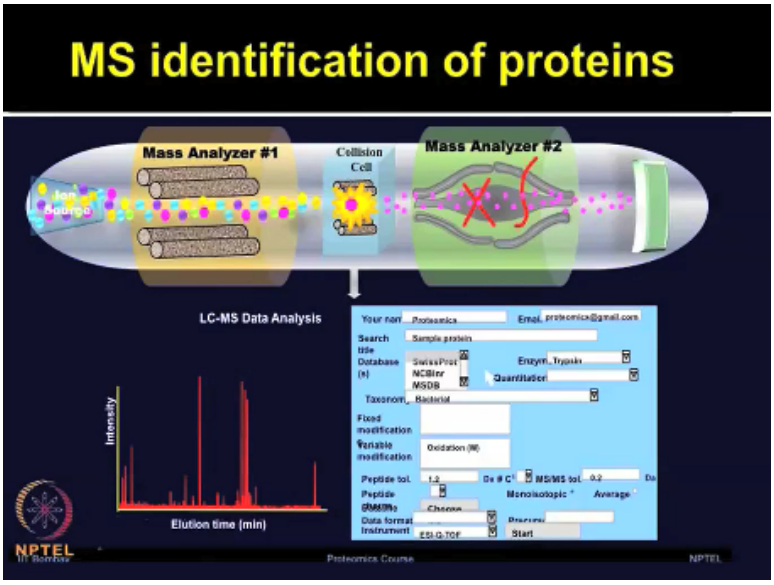

Now these interesting 38 differentially and significantly expressed proteins were excised from the gels and subjected to the multi tough mass spectrometry for analysis. Authors also employed liquid chromatography TAMDEM MS MS system for analysis of these spots and then the data was analyzed by using mascot search engine.

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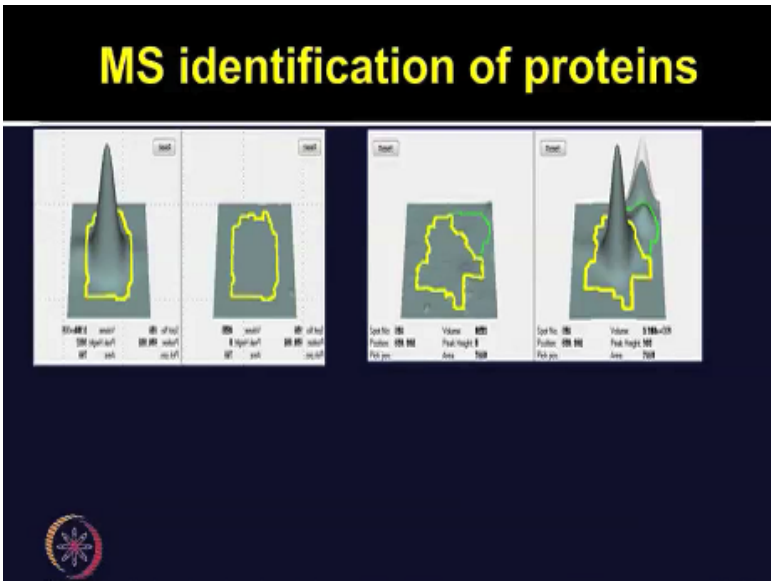

After the identity of these proteins were established the 7 proteins which were not detectable in the healthy controls and only observed in the SARS patient. The identity of those included glutathione peroxidase, Prx II, retinol binding protein, vitamin D binding protein and serum amyloid proteins. They also found that there are 8 proteins which were over expressed and those included pigment epithelium derived factor 2HS like a protein complement factor H related protein and leucine rich 2 glycoproteins.

For a complete list of proteins identified you can refer to this manuscript these are some of the interesting proteins which authors identified.

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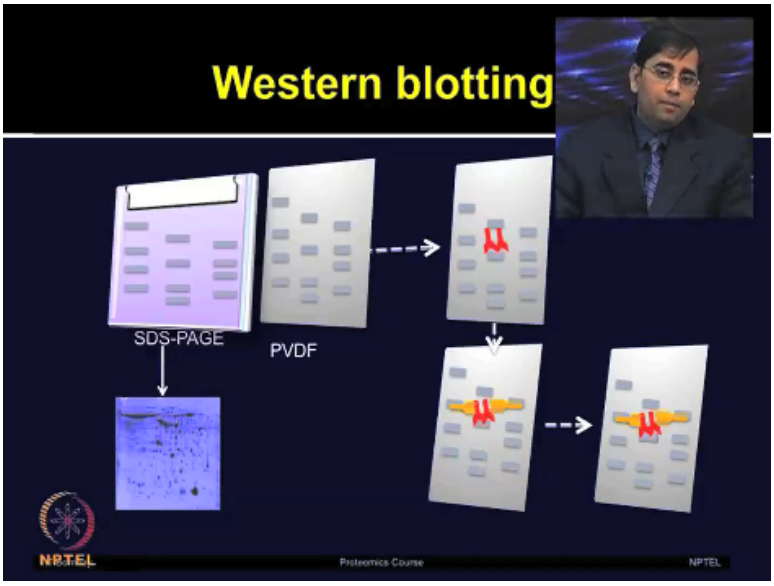

After the identity of these proteins were established then western blot analysis was used to validate few targets 7th identified proteins author found that peroxiredoxin 2 was very interesting. So, they observed than an intracellular protein Prx II exclusively found in the plasma of SARS patients but that was absent in the healthy individual.

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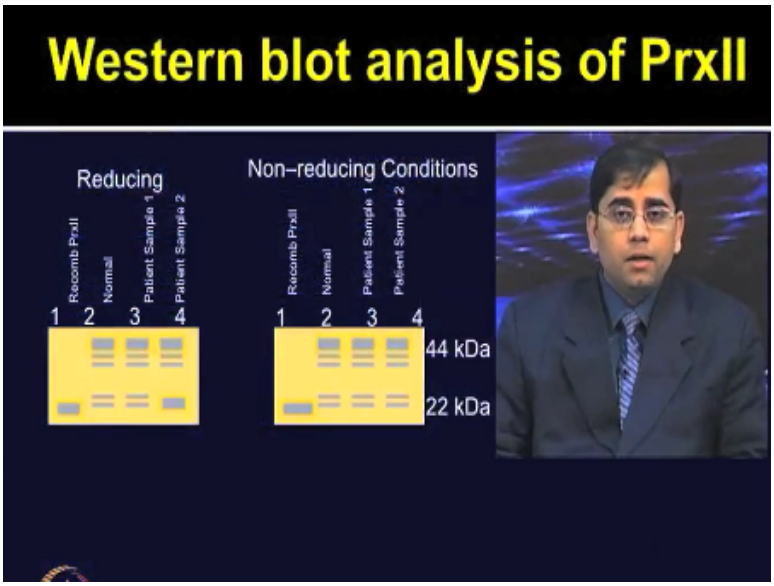

These results demonstrated that Prx II can be present in the monomeric or dimeric form. So, they perform the western blot in both reducing and the non-reducing conditions as you can see in the slide and then they loaded different samples including recombinant peroxiredoxin normal or healthy individual patient sample 1 and patient sample 2. S, the western blot result shown that the Prx II is present in the monomeric form at the 22 kilo Dalton.

As you can see the molecular weight mentioned in the slide in the reducing condition and in the dimeric form at 44 kilo Dalton under the non-reducing conditions. These results demonstrated that 4 out of 20 SARS probable cases and 4 of the 20 SAR suspected cases showed higher up regulation of the plasma peroxiredoxin II.

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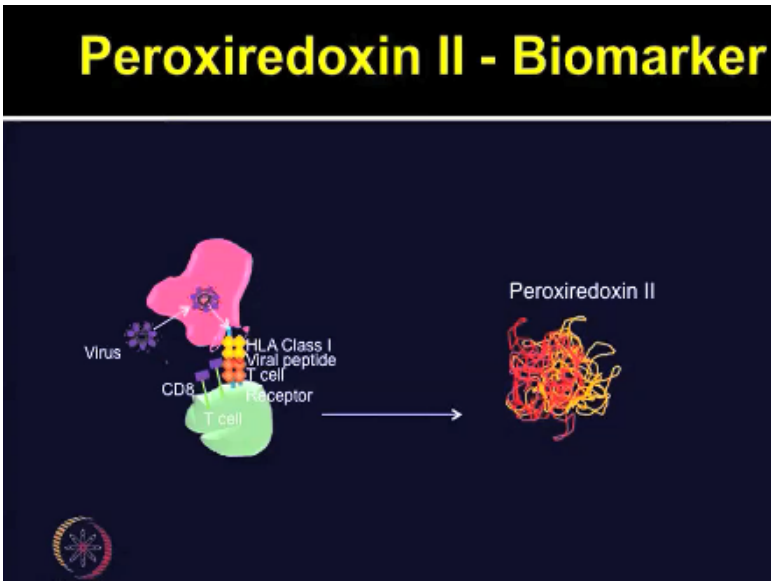

So, among various proteins which were interesting identified from the study a peroxiredoxin II was appeared quite interesting and also because it was validated by the independent technique. So, the level of plasma peroxiredoxin II in patients with SARS was significantly high proteomic analysis and western blot validation suggested that peroxiredoxin II may be used as one of the SARS disease associated biomarker.

They also found that several acute phase proteins or APPs those were differentially expressed.

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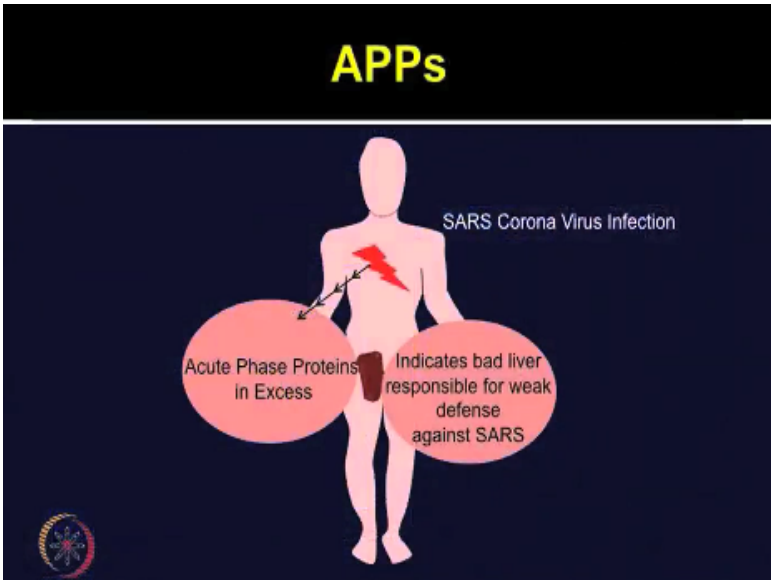

The active innate immune responses and oxidation associated injuries may play a major role in the SARS pathogenesis. The acute phase proteins found in the SARS patients suggested that the

health status of liver in these patients may be affected their difference against the SARS virus infection. So, some of these information authors are able to obtain because of the proteomic analysis of these plasma samples obtained from SARS patients.

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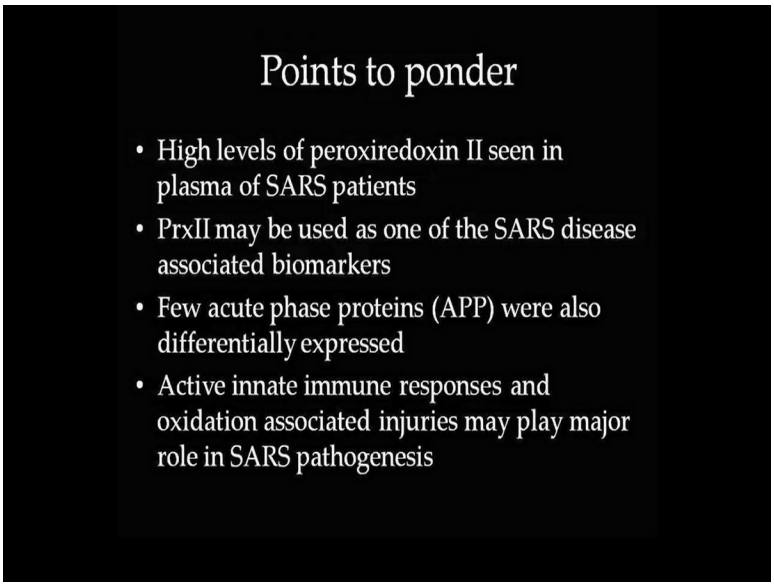

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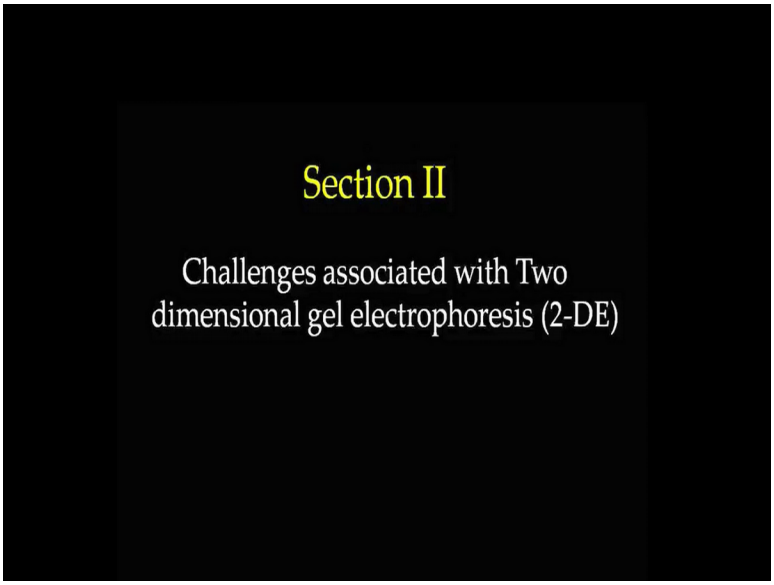

Now in the previous lectures when we talked about obtaining a good 2D gel image.

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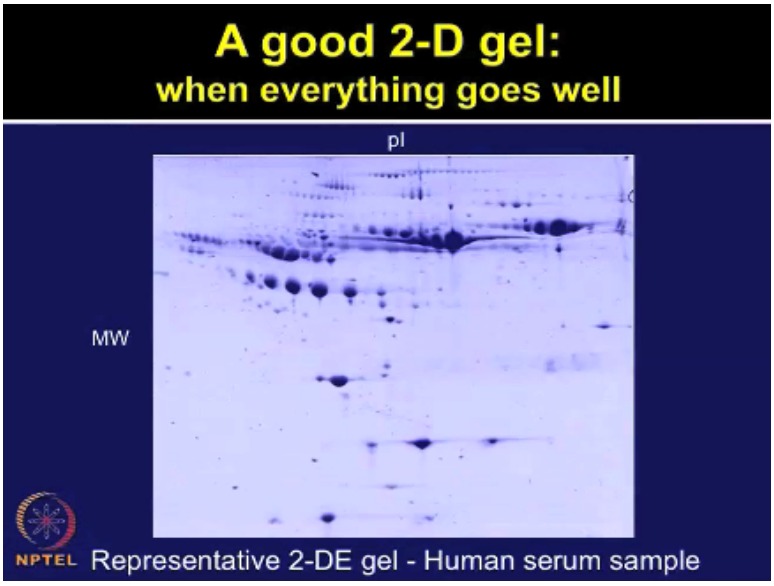

Then you may realize or feel that at the end of that experiment all the 2-D gel image looks very good. So, I am showing you one 2-D gel which is appearing very good and this is something when everything goes very well. So, if a protein separation and steering everything is fine then at the end you should be able to see very good protein separation on the gel. This is a representative 2D gel image shown with the human serum sample.

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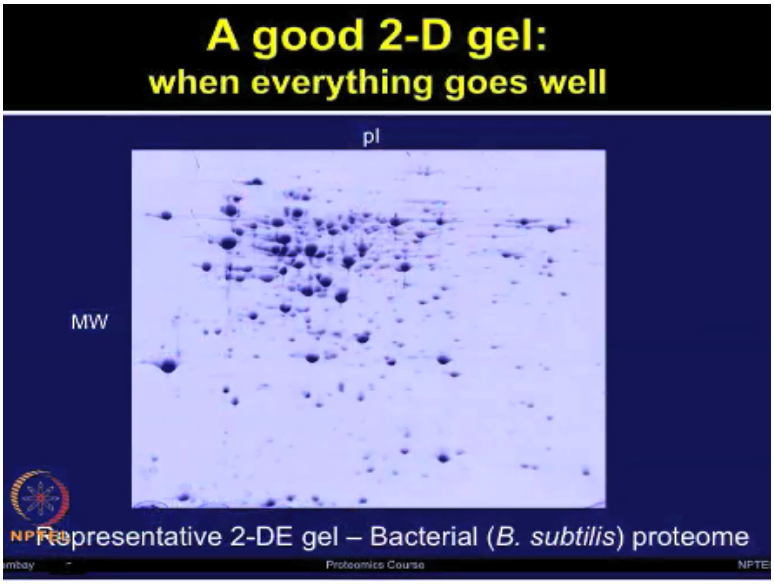

Now I am showing you another good gel obtained from bacterial sample the B subtilis, but often this is not the case many times a different type of issues which could give rise to very bad images the reshoot could be because of the sample preparation method because of regions involved because of Isoelectric focusing different type of parameters involved. And finally, different type

of staining method being used. So, let us talk about some of these issues step by step.

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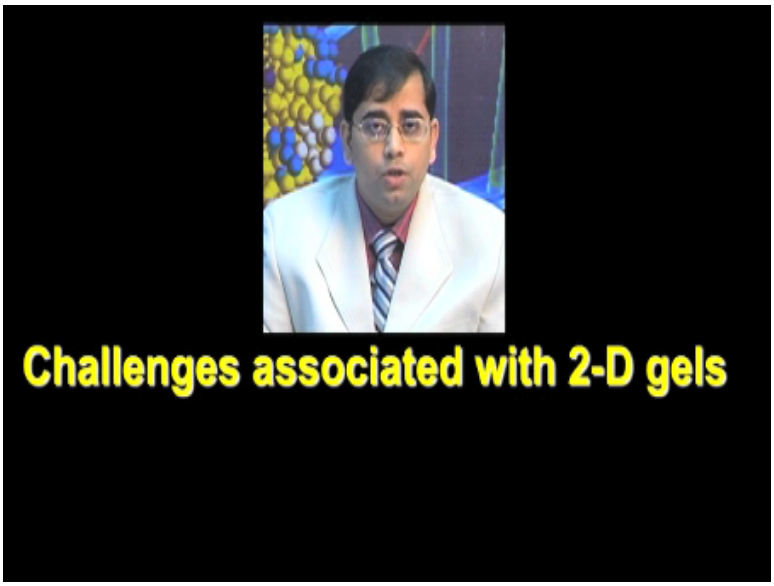

So, there are various challenges which are associated with Two-dimensional electrophoresis mainly the gel artifacts are majorly limitations.

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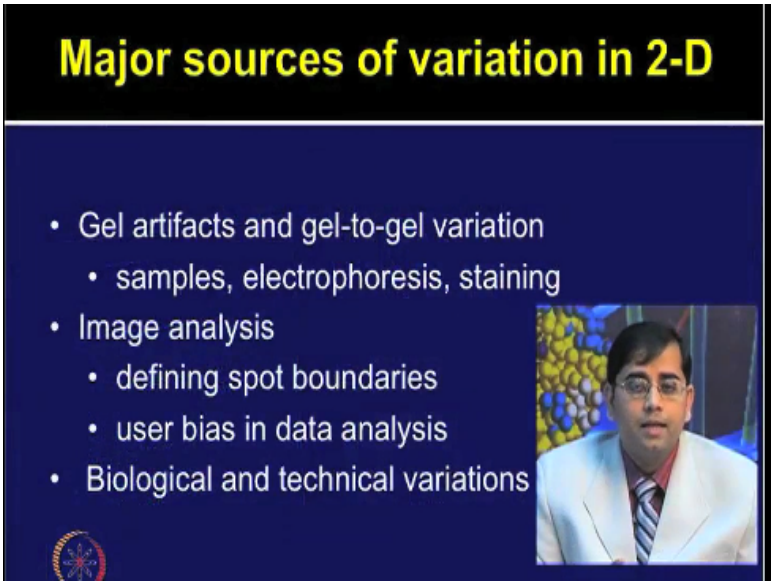

How to compare two gels how to minimize the variations often during the electrophoretic run there is lot of run to run variation. Then you have different type of technical and biological variations. The image analysis that itself is a very challenging process. How to define the spot boundaries how to extract intensity information from those spots often there is a user bias in doing this type of data analysis.

So, there major sources of variations in two-dimensional electrophoresis. I am showing you two problematic gels how even the small mistakes or some issues inherent to your biological samples can give rise to very bad 2-D gels.

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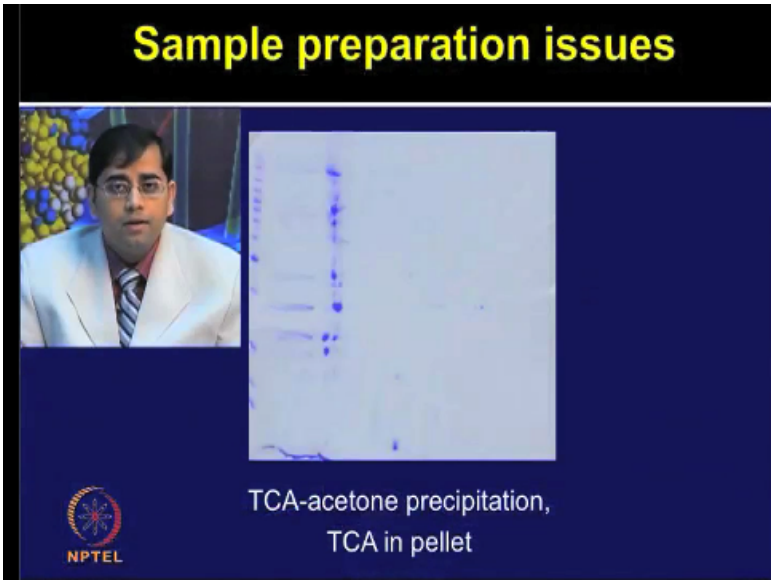

For example, I am showing you a few sample preparation issues in this gel as you can see the TCA acetone precipitation method was followed but there was not good washing performed. So, TCA still remained in the pellet and one can obtain the pattern as shown in this gel.

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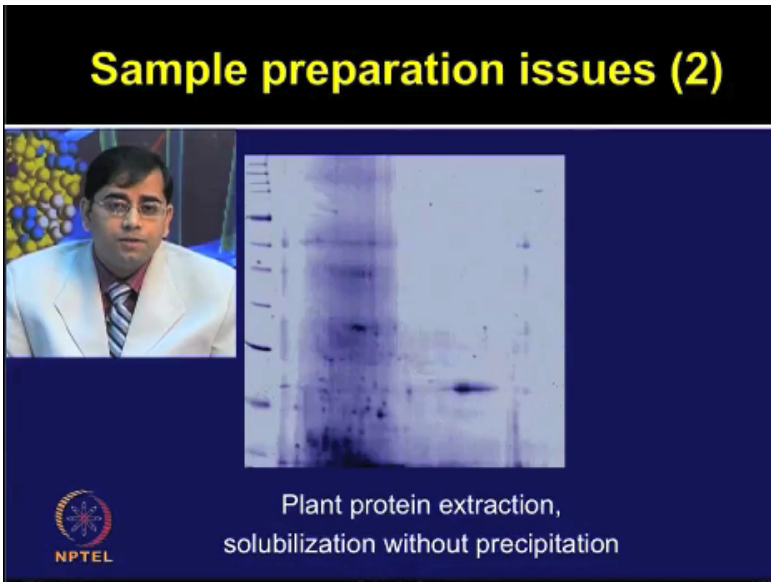

Now if you are performing the plant protein extraction but the solubilization is not sufficient

without precipitation one can see this type of streaking and uneven gel pattern. Now if you have samples which are having proteins in the abundance such as crude serum sample.

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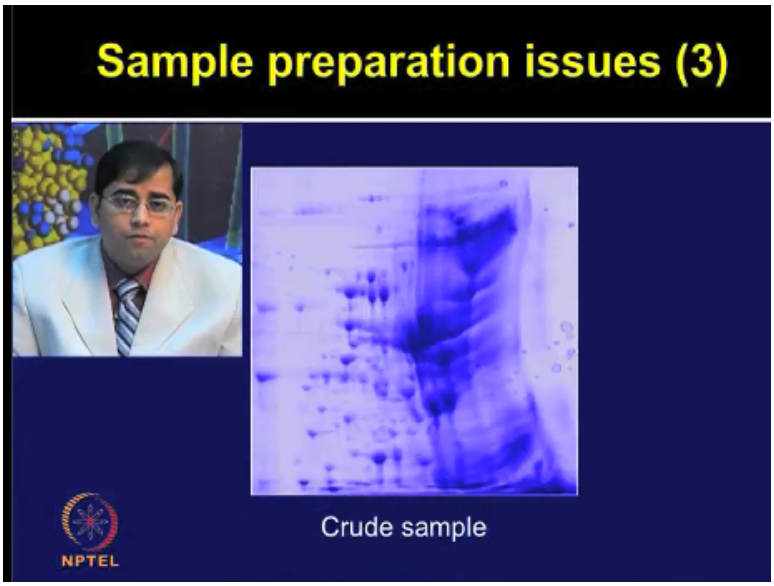

So, these serum samples will show you different interference in the focusing strip because of large salt or rather interfering components present and then you can see these types of bad gels because of the either salt or different type of abundant proteins present in the mixture. Now the sample variation is one problem often the chemical impurities or the chemical ingredients can also give rise to different type of bad gels.

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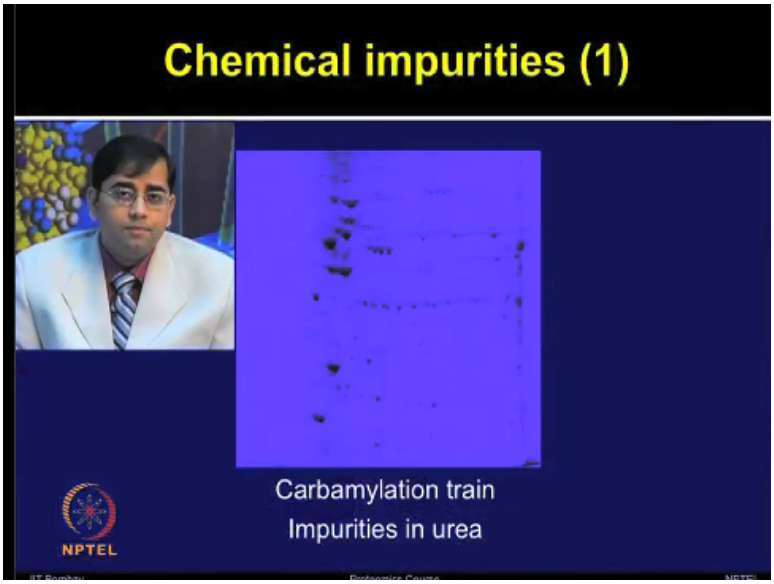

Such as the chemical impurities shown in this gel where the urea is very impure, and one can see

the carbamylation train as shown in this gel. If the TEMED is very old often the TEMED is very small amount of TEMED is used.

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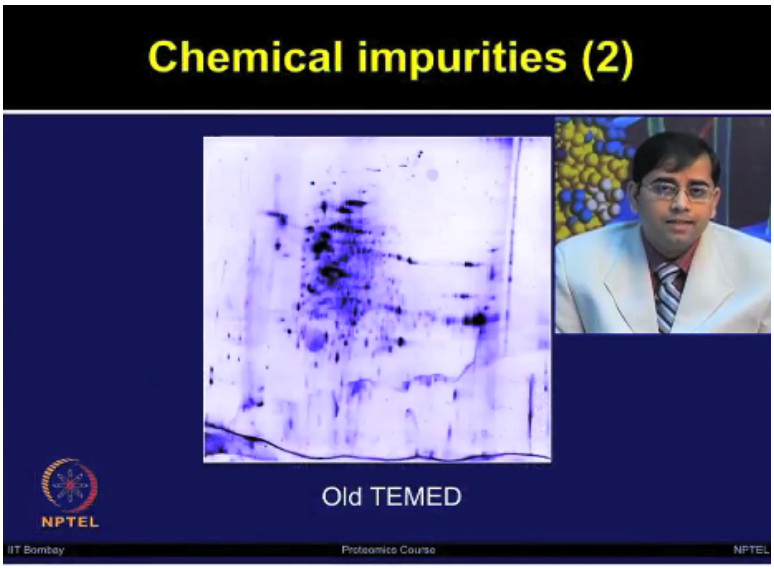
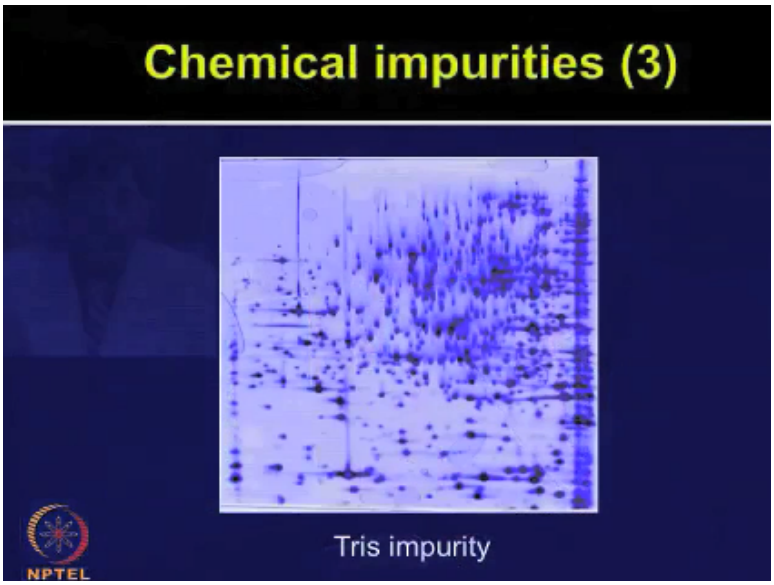


In making the second dimensional gel as SDS page. So, people often use that bottle for very long time and if TEMED is very old you can see some bad pattern on the gel. Something similar to as shown in this image.

(Refer Slide Time: 26:10)



Now if your chemical such as Tris is not a very good quality it is impure again that will show some artifacts in the gel. It is streaking that is very oftenly seen.

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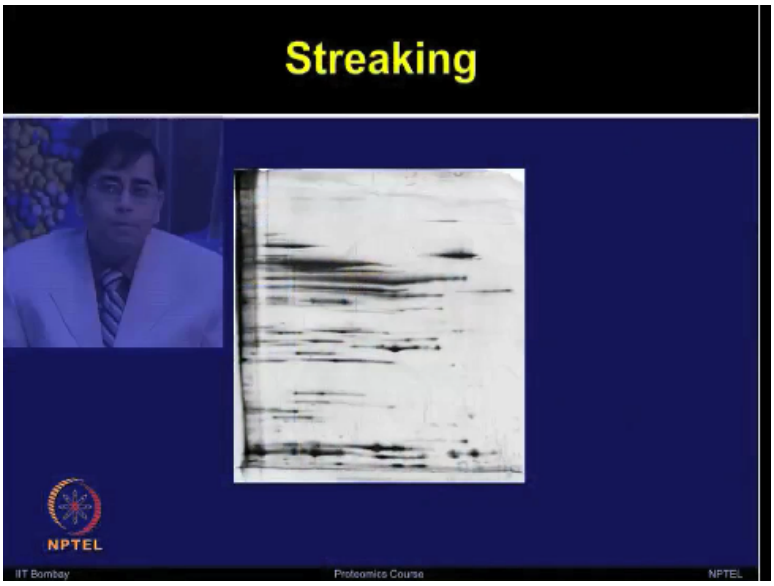
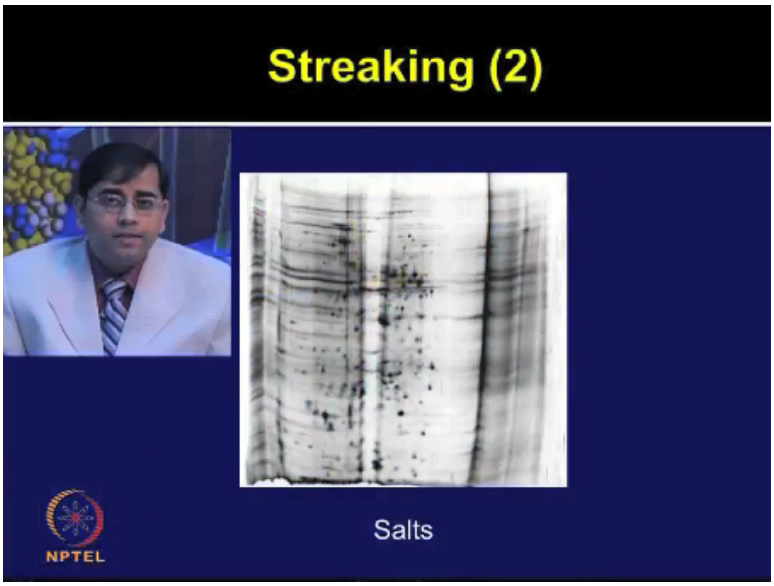


Because of different type of problems which could be associated whether it is coming from the sample preparation or because of different type of interfering components often you have nucleic acid or carbohydrates is still present in the mixture. The extraction method was not sufficient to eliminate all of those impurities many times SARS and different other interfering components are also present which interfere in the IEF process.

And one can see the streaking pattern after looking at the staining of the gels. So as shown in this gel.

(Refer Slide Time: 27:04)



The presence of salt can be interfering and may result into vertical streaking as shown in this gel.

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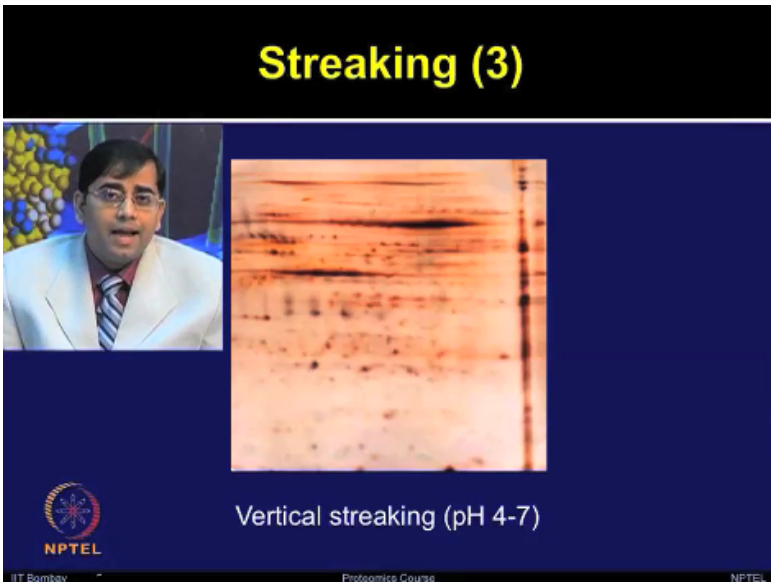

If you are using narrow pH gradient strip such as 4 to 7 pH strips, then the vertical streaking can appear because all the proteins beyond 7 PI will stuck together in that region and one can see the word vertical streaking.

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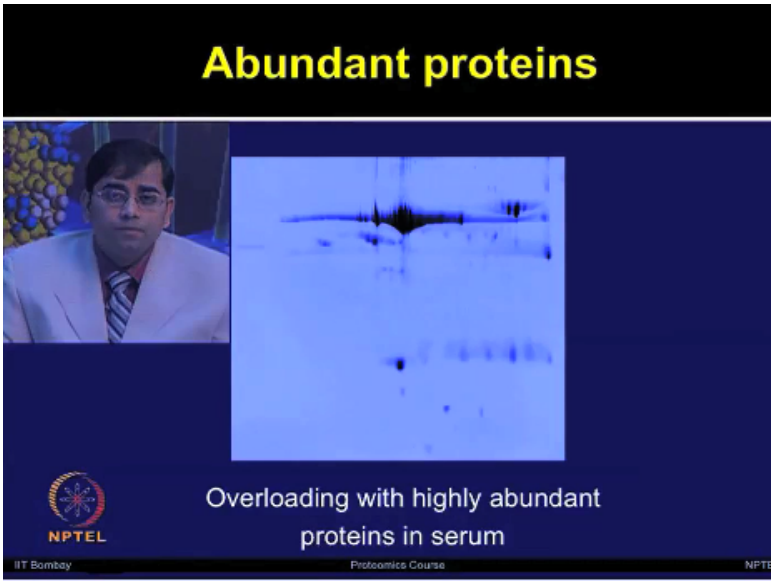

Now abundant proteins as a brief we briefly talked last time sometime crude samples to contain highly abundant proteins and SARS. So, abundant proteins are one of the major interfering components which can be easily seen on the gel. If you have serum sample, there are many interfering components present in the serum including some abundant proteins such as a serum albumin.

Now in the plants one can expect rubisco as the one of the very large protein present in the plant leaves. So, these abundant proteins they mask many smaller protein and create problem in doing the two-dimensional electrophoresis because of IEF process as well as when you stain on the gel you can see different type of streaking as well as different the whole region is masked because of the abundance of that protein.

So, in the Still it is shown that the human serum protein contains high abundant proteins which is visible in this area know there are various ways one can overcome these limitations and as in the previous class of the protein if extraction and sample preparation. We have talked about how to overcome these limitations how to remove the highly abundant proteins. So please refer to that lecture and one can overcome the limitation.

Now after protein extraction and IEF process is done one needs to equilibrate this stip.

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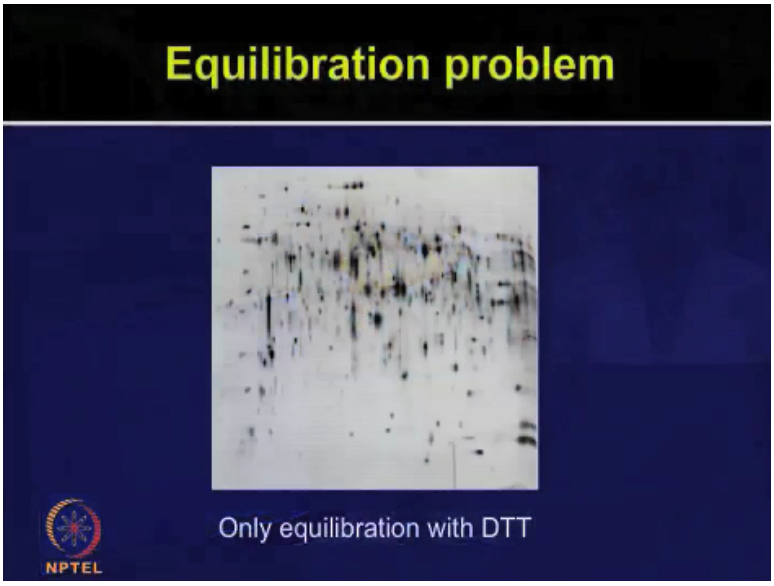

Equilibration process itself can create some problems for example if you have performed only first equilibration with DTT and forgot to do the second dimension the second equilibration prior to doing the second-dimension separation. Then you can see some pattern similar to as shown here that only equilibration with the DTT many times to people think that by increasing the time of doing equilibration probably the gel quality may appear better but that is not the case.

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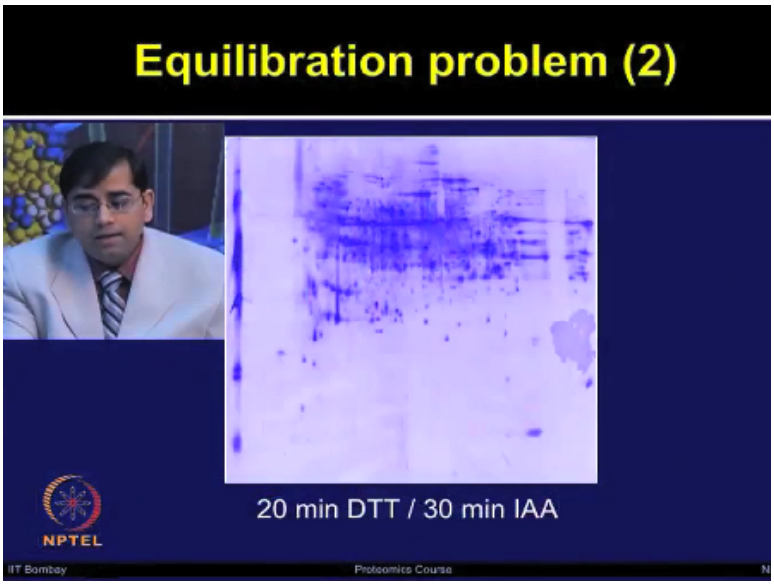

In this gel it is shown that by increasing the equilibration time to 20 minutes of DTT and 30 minutes of iodoacetamide. The gel pattern looks very problematic and that is because of the equilibration excess. Now IEF process this very crucial many times during the IEF itself.

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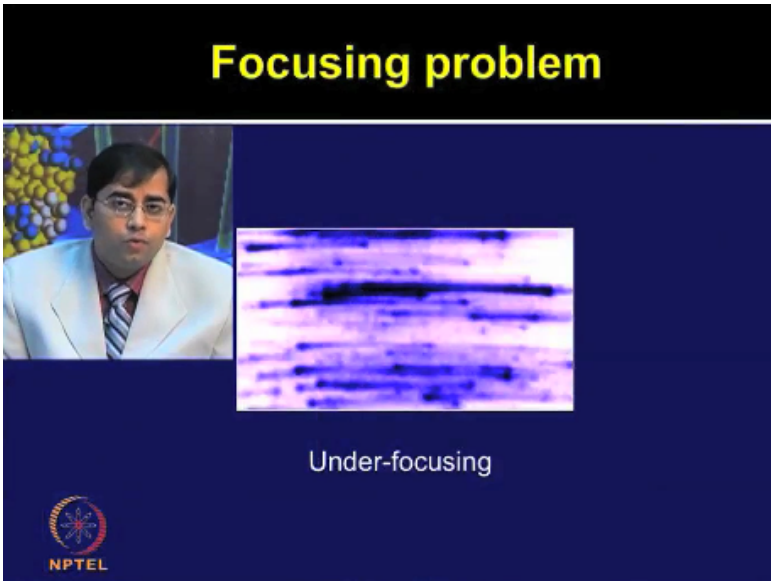
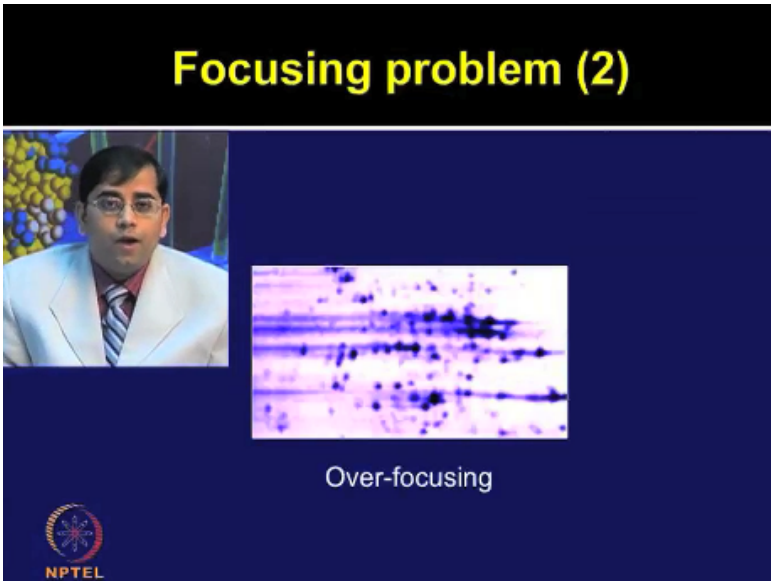


One can monitor the problems if you have a software which is showing you how the run is progressing often if your setting is not correct it is insufficient for the complete focusing it may result in the under focusing as shown in this image here for the under focusing.

(Refer Slide Time: 30:43)



Now if you run too much voltage and the overall volt hour is very much very high then it may result in to over focusing. So, optimizing a focusing protocol for the appropriate duration and overall volt hour is very important. So, there are different type of sorts of variations can be introduced from the biological as well as technical means.

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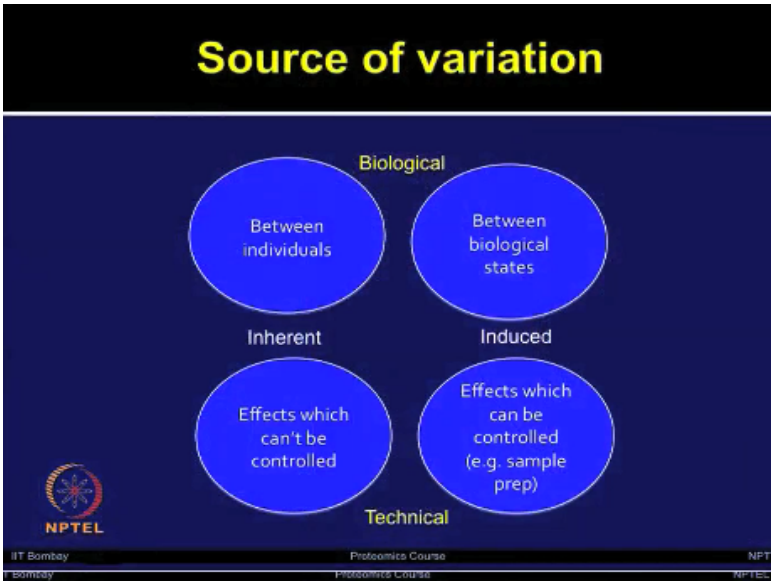

There are different biological variations on which there is no control if you are doing some clinical studies then even if you have done proper age and gender match is still there will be some variations due to each individual's uniqueness. So, those biological variations are very tough to eliminate. But then there are a lot of technical variations which can be improved during the sample preparation during the IEF settings during intubation steps.

So, all those steps may result into large variations. So, in two-dimensional electrophoresis when you are separating your control and treatment gels on two different gels and you have so many variations coming from both biological and technical variants the analysis and the confidence in the data becomes very very questionable.

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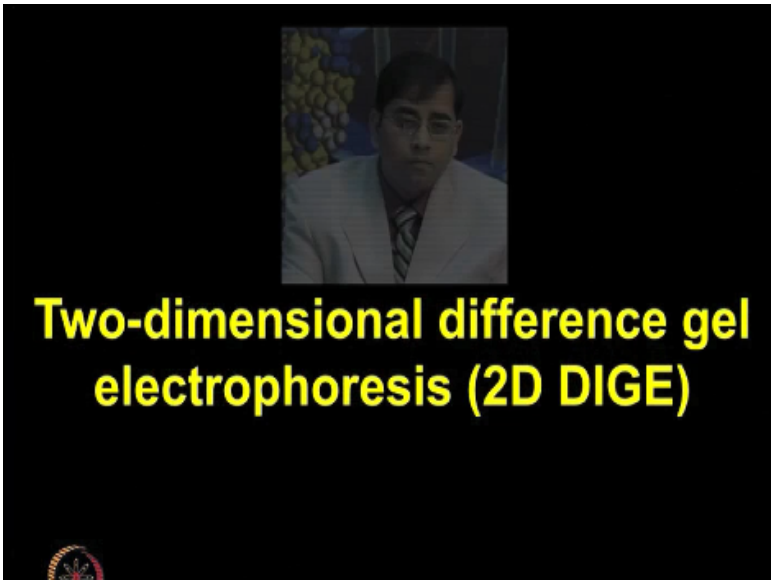

To overcome those limitations, the new approach the advanced electrophoresis method two-dimensional difference gel electrophoresis is 2D DIGE have emerged.

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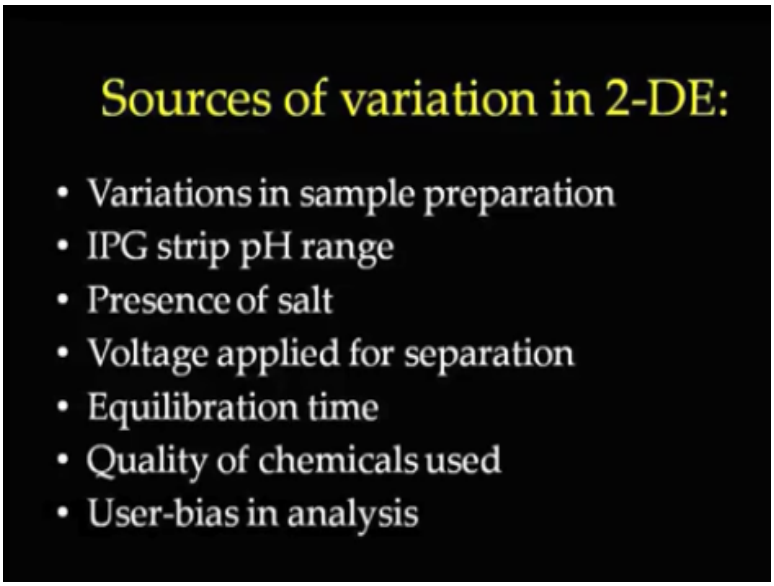

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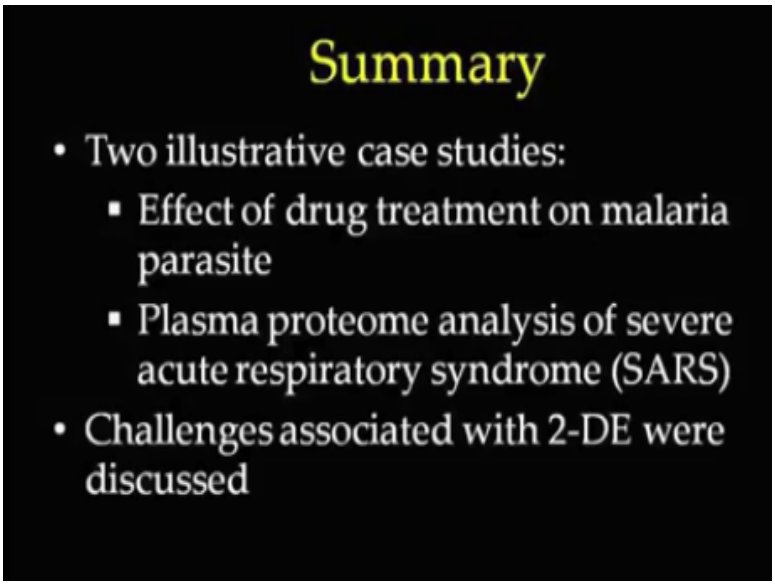

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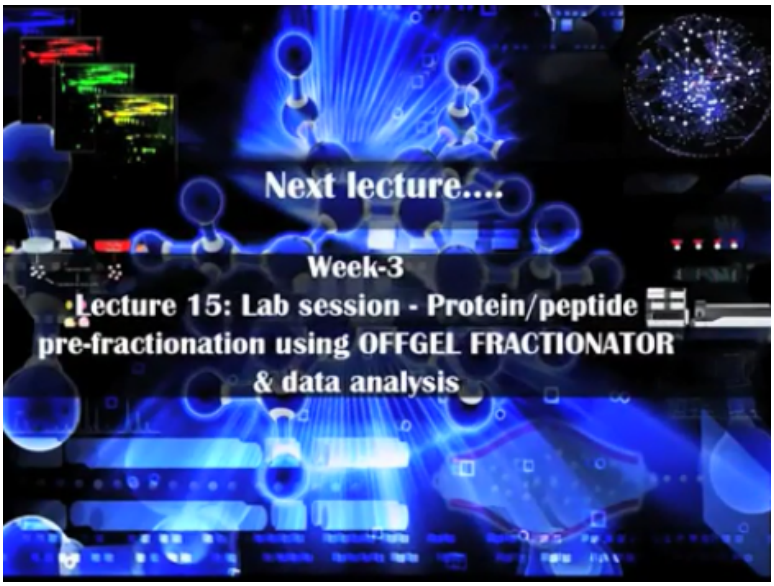

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REFERENCES

- Makanga et al. Towards a proteomic definition of CoArtem action in Plasmodium falciparum malaria. Proteomics. 2005, 5, 1849
- Chen et al. Plasma proteome of severe acute respiratory syndrome analyzed by two-dimensional gel electrophoresis and mass spectrometry. Proc Natl Acad Sci USA. 2004, 101, 17039
- Byrne et al. 2D-DIGE as a Strategy To Identify Serum Markers for the Progression of Prostate Cancer. J Proteome Research. 2009, 8, 942-57
- Issaq H, Veenstra T. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): advances and perspectives. Biotechniques. 2008, 44, 697-8, 700.