Proteins and Gel-Based Proteomics
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Mod 04 Lecture Number 16

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Topics to be discussed today

- Case studies of Two dimensional gel electrophoresis (2-DE):
  - Effect of drug treatment on malaria parasite
  - Plasma proteome analysis of severe acute respiratory syndrome (SARS)
- Challenges associated with Two dimensional gel electrophoresis (2-DE)

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Section I

Two dimensional gel electrophoresis (2-DE) case study

In today's lecture we will talk about …

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… Applications of two-dimensional electrophoresis If you recall the previous lecture, we started discussing about some case studies about two-dimensional electrophoresis applications. As you have studied in this module what are different types of workflow involved in performing two-dimensional electrophoresis 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, preparing the strips for second dimensional separation and then we separate the proteins from the SDS PAGE based on the molecular weight followed by, … stain the gels to see the visualization 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 same regardless of whatever application one wants to use in their different types of biological questions. I started discussing about two-dimensional electrophoresis applications in the last class. I gave you an overview of the 2DE at that time.

So, let's continue our today's lecture from the same theme and let's discuss some more case studies on how people have employed two-dimensional electrophoresis, the power of this technique to resolve thousands of proteins and compare those for various differential proteomic applications.

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Topics to be discussed today

- Case studies of Two dimensional gel electrophoresis (2-DE):
  - Effect of drug treatment on malaria parasite
  - Plasma proteome analysis of severe acute respiratory syndrome (SARS)
- Challenges associated with Two dimensional gel electrophoresis (2-DE)

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. We will then take a study on the plasma proteome analysis of SARS virus.

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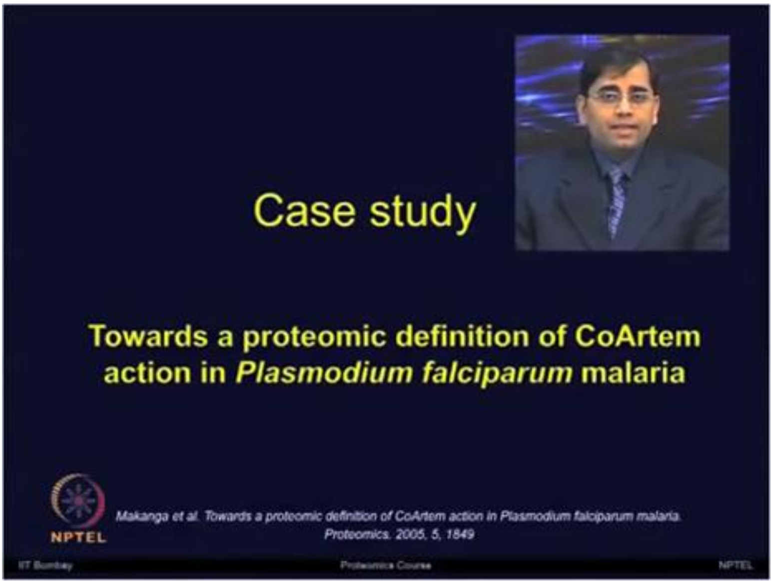

So let's start with the first case study towards proteomic definition of CoArtem action in Plasmodium falciparum malaria, a study by Makanga et al in 2005.

So as you know, each year hundreds of millions of new malaria 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 lots of deaths are happening and the malaria problem is still posing challenges for its control.

The anti-malaria therapy of chloroquine and pyrimethamine, these have not been able to control the mortality rate because of the anti-malarial drug resistance development. So, therefore there is urgent need for identifying new drug targets as well as understanding course of action of these drugs by applying various types of high throughput techniques.

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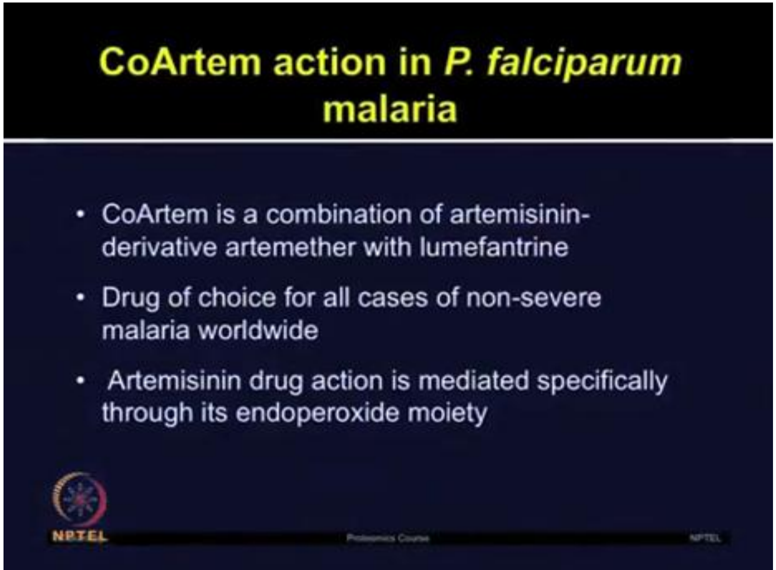

So in this paper, authors have discussed how 2 different drugs which are effective for the anti-malarial can be studied for looking at the proteome changes in the Plasmodium falciparum parasite. So CoArtem is a combination of artemisinin derived artemether with lumefantrine. How these 2 drugs behave and how the proteome changes occur due to the action of these 2 drugs were studied in this paper.

Authors applied proteomic approaches, the two-dimensional electrophoresis to study the proteomic alteration of each of these drugs.

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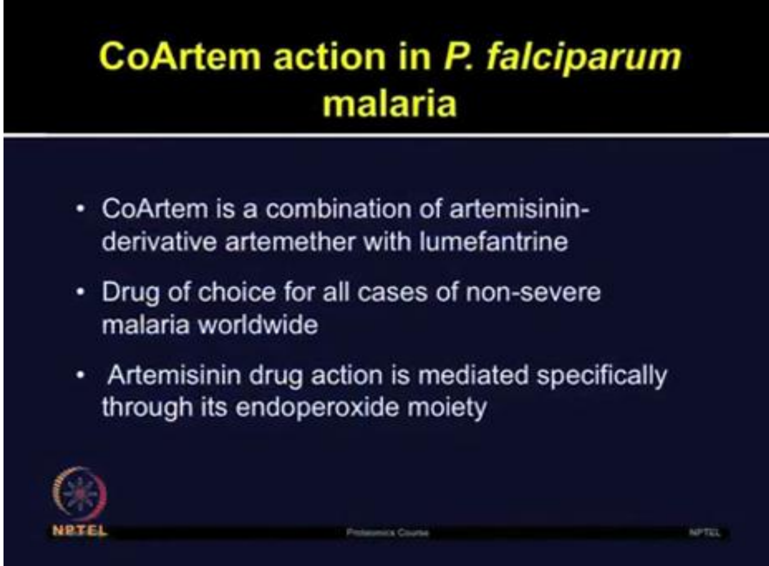

So these drugs are applied as a drug of choice for all cases of non-severe malaria worldwide. The artemisinin drug action is mediated specifically through its endoperoxide moiety. However the more detailed mechanism of action of these drugs are still unknown.

So the purpose of this 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 the alterations in parasite proteome which were induced by each of these drugs.

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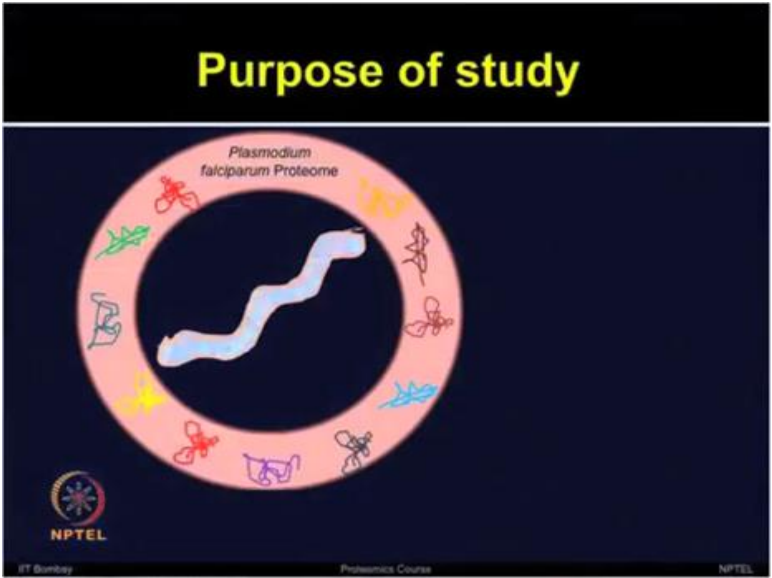

To obtain the insight of the proteomic alteration, they separated the proteins from the two-dimensional electrophoresis -gels and compared the response of these proteomic alterations

based on these two drugs. And then they identified certain proteins which were either commonly expressed due to these drugs or they were differentially expressed due to these drugs.

Certain proteins were found to be commonly up-regulated due to both of these drugs and certain proteins have the different patterns.

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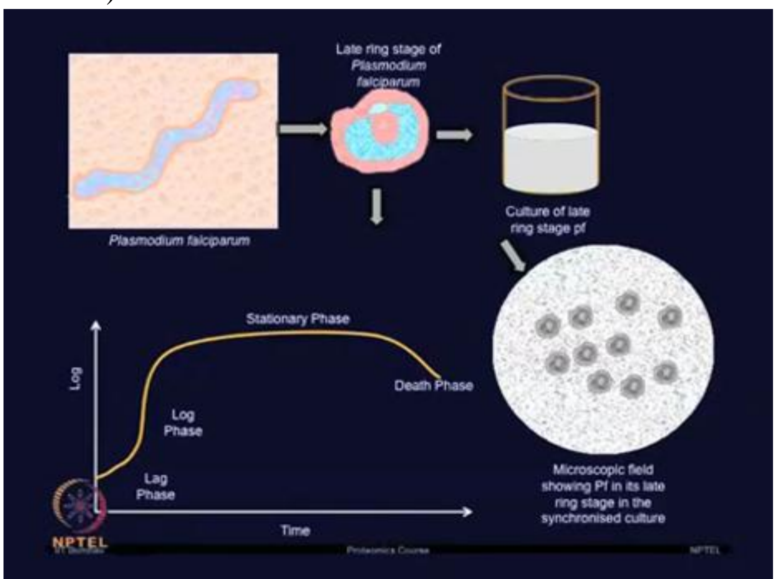

But before looking at the proteomic alterations, authors first determined IC 10, 20, 50 and IC 90 values for both the drugs ARM and LUM. We will use the abbreviations now for artemisinin and lumefantrine. And effect of this concentration of drugs on parasite growth over 24 hours was characterized.

As you can see the growth curve in this slide, synchronized ring shaped parasite cultures 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 looked for the proteomic alterations. So first of all they did the fractionation of the P. falciparum proteome,

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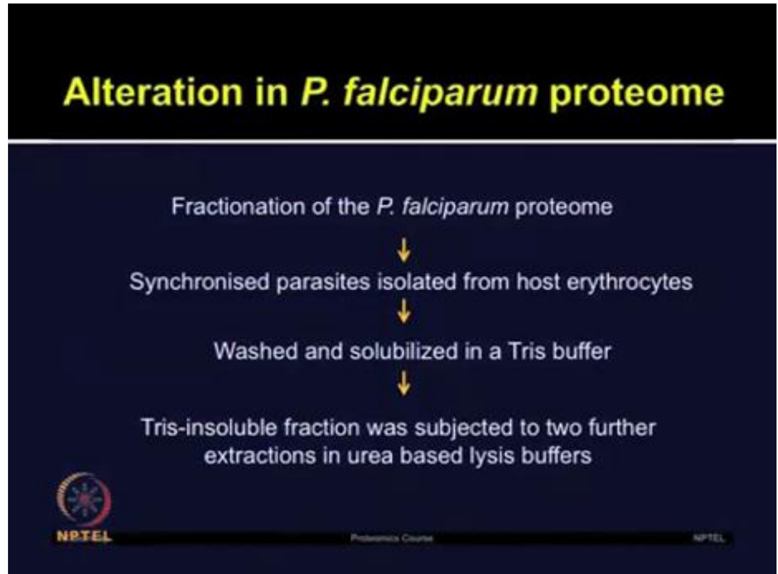

Synchronized parasites which were isolated from the host erythrocytes, wash those initially and solubilze that in a Tris-buffer recipe. The Tris-insoluble fraction was further subjected to extraction in the urea-based lysis buffer.

Once 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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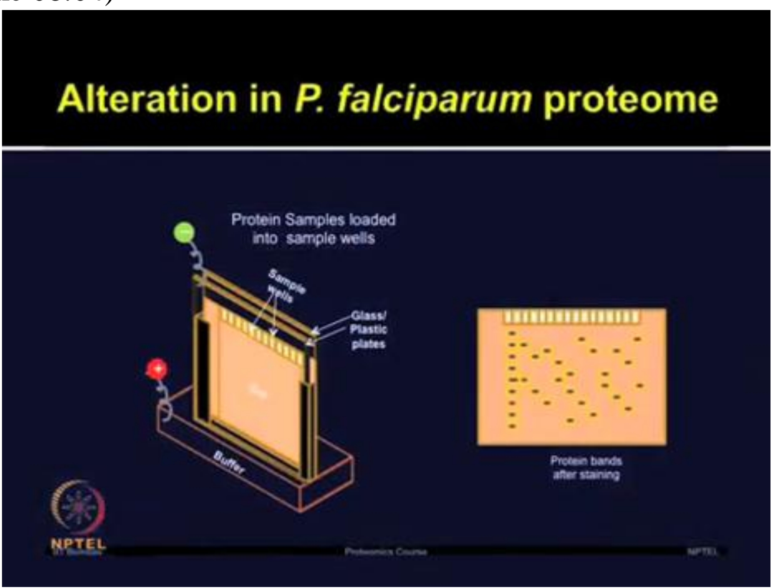

After the IEF was done, they equilibrated these IPG strips and then applied that on 12.5% of the vertical SDS gel. After the second dimension separation based on the molecular weight, then these gels were stained with the silver or Coomassie brilliant blue stains.

So by employing two-dimensional electrophoresis and comparing the gel images by using the PDQuest software, authors are able to see that there is differential proteomic response which is drug-specific.

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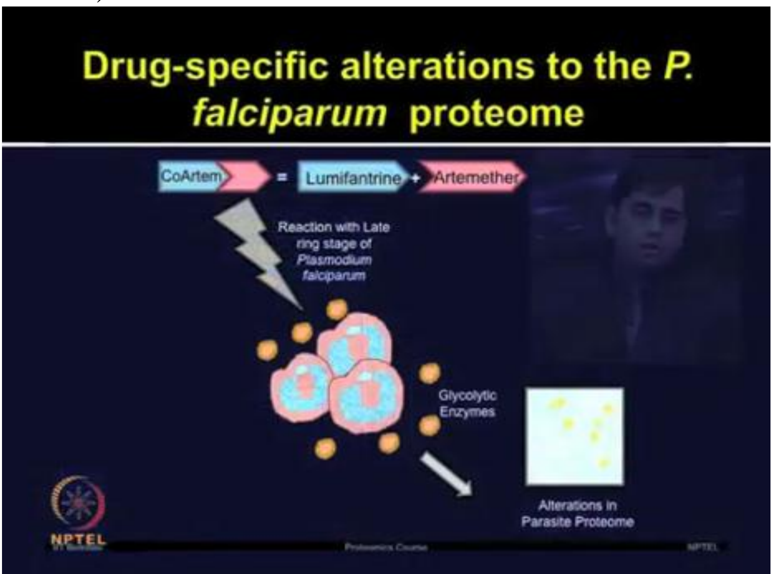

Quantitative analysis of the altered protein expression levels following exposure to the ARM and LUM were analyzed and then those protein spots which were differentially expressed and statistically significant were further subjected to the mass spectrometry based analysis.

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 and lumefantrine drugs

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Altered protein expression levels following drug exposure

	Artemether	Lumefantrine
Membrane associated calcium binding protein	U	U
Aspartic proteinase (HAP)	U	U
HSP60, 70, 90	U	U
Enolase	D	U
Fructose biphosphate aldolase	D	U
Phophoglycerate kinase	D	U

. Certain proteins were identified, few of those showed 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 were up-regulated 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 up-regulated due to both the drug treatments. There are certain proteins such as Enolase, fructose bisphosphate aldolase and phosphoglycerate kinase, these proteins were down-regulated in Artimether treatment and up-regulated in the lumefantrine treatment.

So interestingly, the ARM treatment resulted in the more than 3 fold down-regulation of the glycolytic enzymes such as Enolase, phosphoglycerate kinase, fructose bisphosphate aldolase and glyceraldehyde-3 phosphate dehydrogenase...

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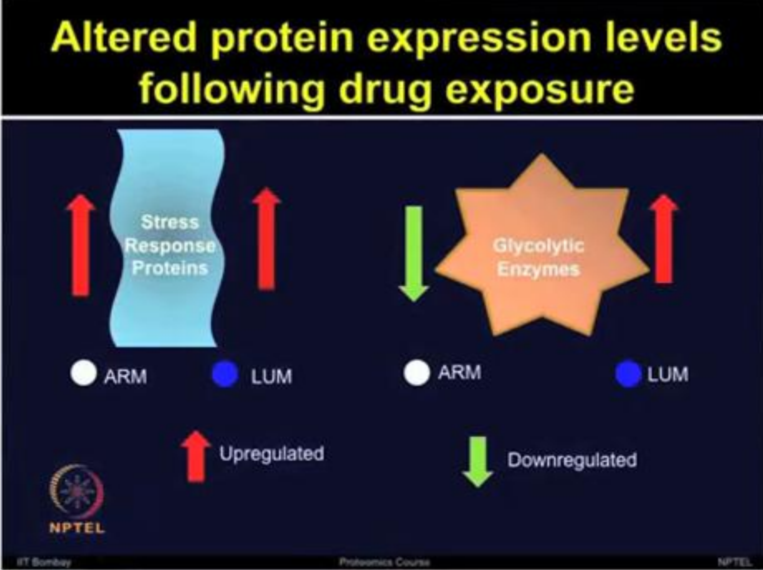

The expression of the same enzymes were also up-regulated more than 3 fold due to the lumefantrine treatment

However certain proteins such as the stress responsive proteins, the heat shock proteins which were commonly induced due to either of these drug treatments which looks like a general stress response as compared to very unique response to the given drugs.

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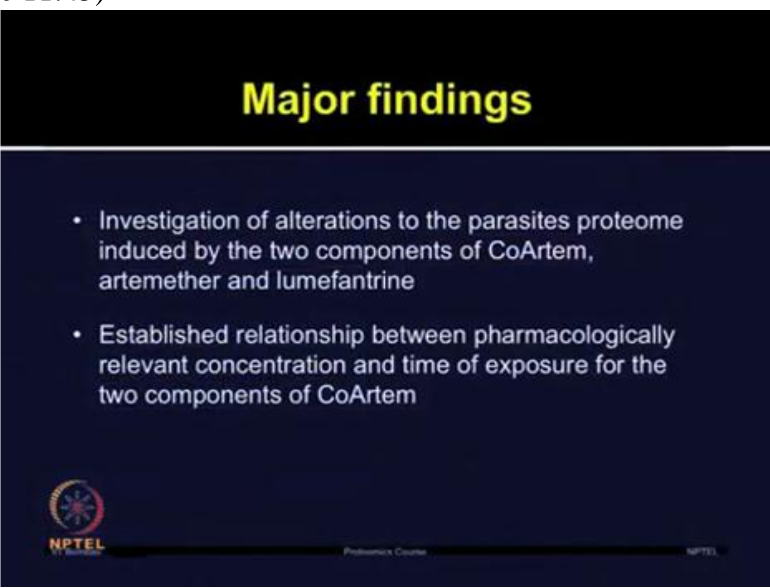

So from this study the major findings were that the authors successfully investigated alterations of parasite proteomes induced by 2 components of CoArtem, artemether and lumefantrine. By using proteomic approach, they investigated specific and non-specific effects of 2 anti-malarial drugs in pharmacological relevant conditions.

Expression of certain proteins were quite interesting including a membrane bound calcium binding protein which was up-regulated due to Artimether and lumefantrine treatment. The study also established the relation between the pharmacologically relevant concentration and time of exposure for the two components of CoArtem.

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Points to ponder

- Alterations of the parasite proteome induced by two components of coArtem, artemether and lumefantrine were invested
- Effects of two anti-malarial drugs in pharmacological relevant conditions were studied
- Membrane bound calcium binding protein was seen to be up-regulated due to ARM and LUM treatment

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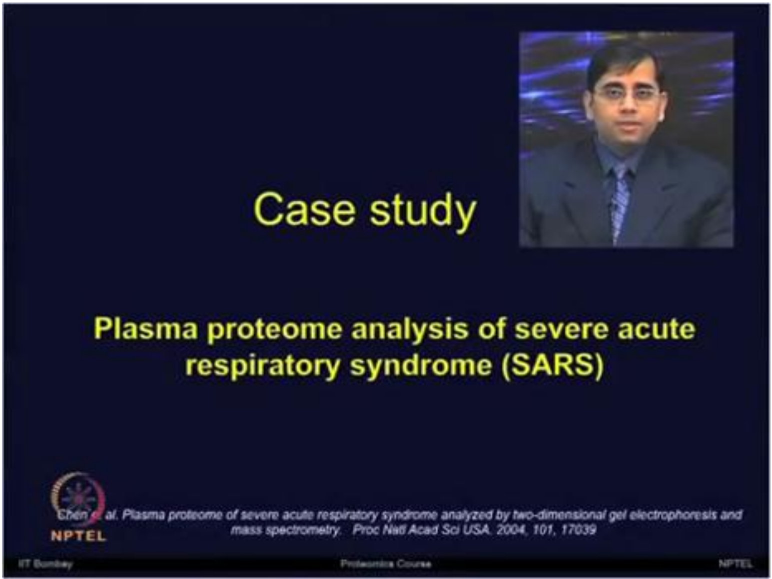

Let’s now move on to Case Study 2, plasma proteome analysis of Severe Acute Respiratory Syndrome SARS, study by Chen et al in 2004. So the purpose of study was to perform a comprehensive plasma proteome analysis…

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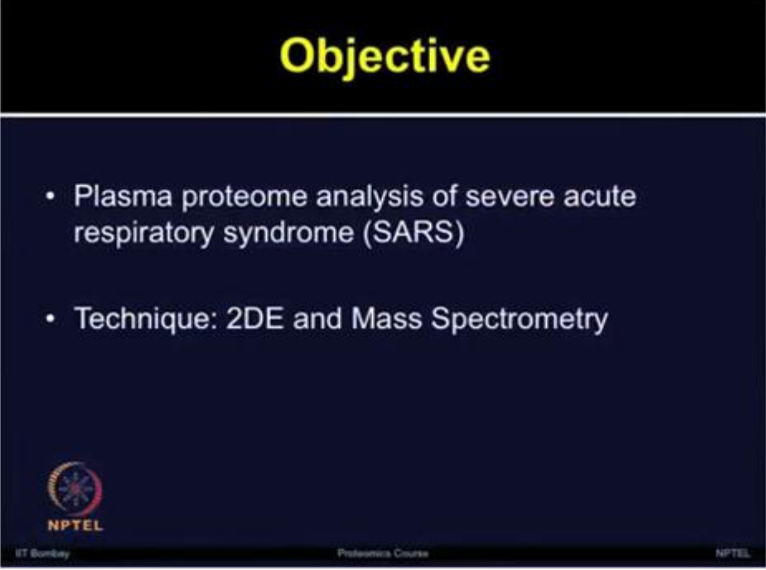

…of Severe Acute Respiratory Syndrome and its comparison with the healthy individuals In this study authors employed conventional two-dimensional electrophoresis, analyzed those images from the treatment with the controls and identified the differentially expressed proteins by using mass spectrometry techniques including MALDI TOF/TOF and LC-MS/MS. Finally the interesting identified proteins were validated from techniques such as Western blots.

So if you remember 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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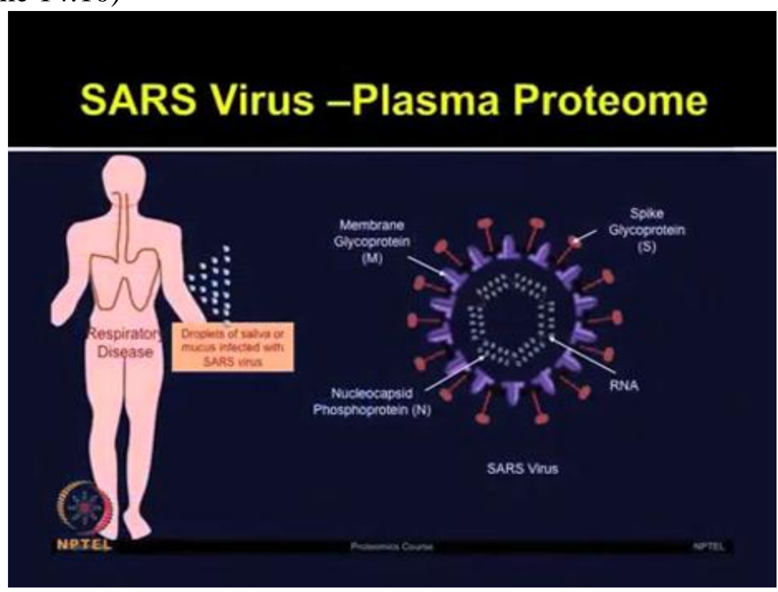

The genome sequence of SARS virus was already known and the structure of main protease and receptors were also known. However the pathogenesis of SARS was not very clearly understood. Since serum or plasma, they provide a very valuable sample to identify the targets for diagnostic, prognostic and therapeutics.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

For the plasma proteome analysis authors explored the possible pathogenetic mechanisms of progression of SARS by analyzing plasma proteins of 22 different plasma samples which were obtained from the 4 SARS patients and 6 healthy controls.

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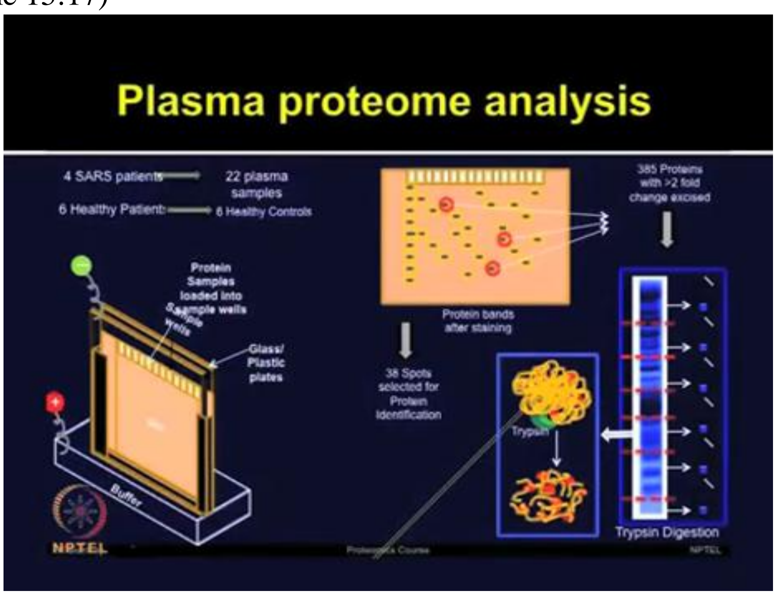

Authors analyzed plasma proteome by using two-dimensional electrophoresis on 4 to 7 pH immobilized pH gradient strips and they stained the gel with the sensitive stain. In the slide you can see, the steps involved in the traditional two-dimensional electrophoresis followed by the mass spectrometry.

Both the controls and the treatments, the healthy individuals as well as the patients suffering from SARS plasma was separated, and then proteins were resolved on the 2D gels. Differentially expressed proteins were further subjected to the in-gel trypsin digestion followed by mass spectrometry.

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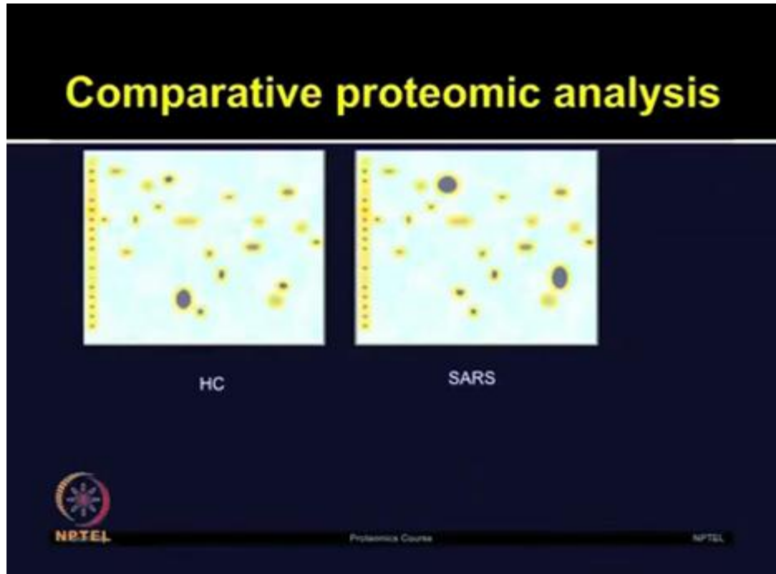

So the complete proteomic analysis of these 2D gels revealed that 38 protein spots were differentially expressed. More than 2 fold change. And out of these spots there were 30 proteins which were up-regulated, 35 were up-regulated, 3 were down-regulated.

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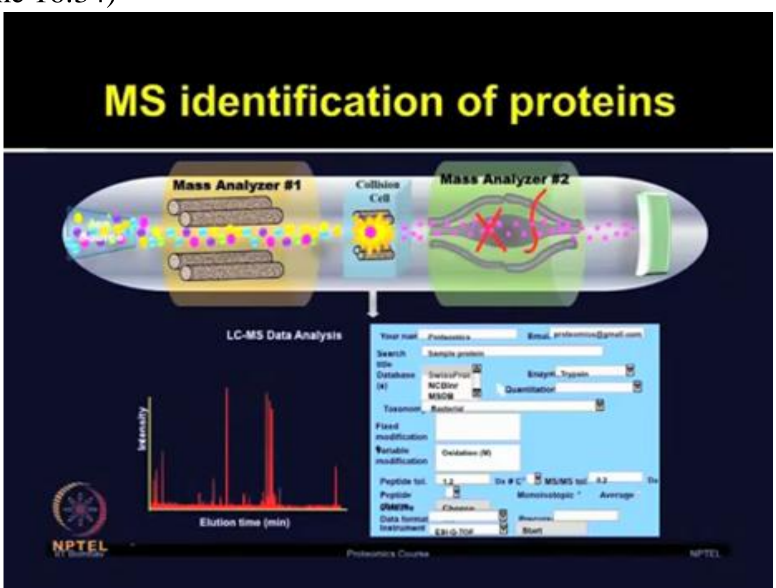

Now these interesting 38 differentially and significantly expressed proteins were exiged from the gels and subjected to the MALDI TOF mass spectrometry for analysis. Authors also employed liquid chromatography, Tandem MS/MS system for analysis of these spots. And then data was analyzed by using Mascot search engine.

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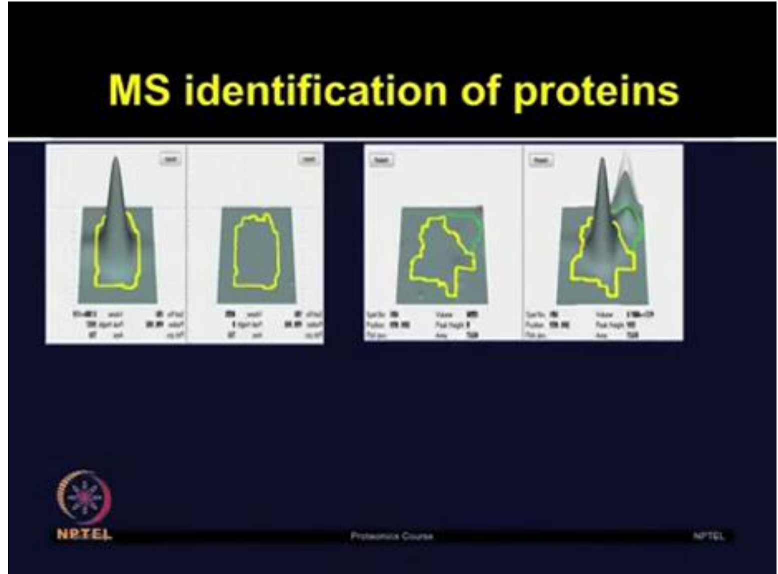

After the identity of these proteins were established, the seven proteins which were not detectable in the healthy controls and only observed in the SARS patients, the identity of those included glutathione peroxidase, Prx2, retinol binding protein, Vitamin D binding protein and Serum amyloid A proteins.

They also found there are 8 proteins which were over-expressed and those included pigment epithelium derived factor, 2-HS-glycoprotein, complement factor h related protein and leucine-rich 2 glycoprotein. For complete list of the proteins identified, you can refer to this manuscript. These are some of the interesting proteins which authors identified.

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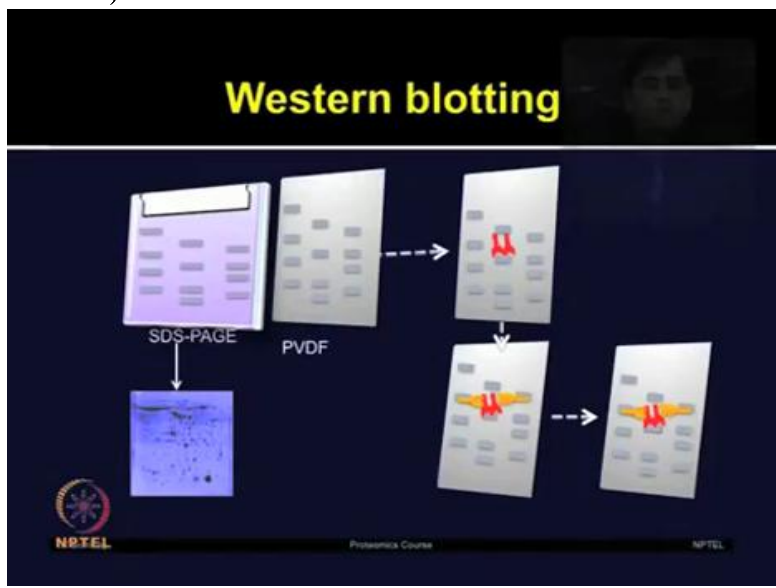

After the identity of these proteins was established then Western Blot analysis was used to validate few targets.

In identified proteins, authors found peroxide hydroxyzine 2 was very interesting. So they observed that an intracellular protein Prx2 excessively found in the plasma of SARS patients that was absent in the healthy individuals.

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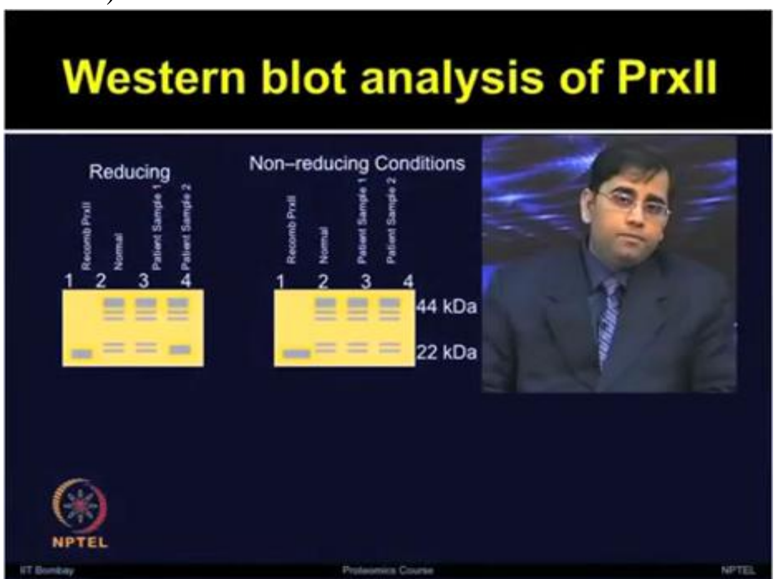

These results demonstrated that Prx2 can be present in the monomeric or dimeric form. So they performed the Western Blot in both reducing and the non-reducing conditions as you can see in the slide, and then they loaded different samples including recombinant peroxiredoxin.

Normal or healthy individual, patient sample 1 and patient sample 2. So the Western blot result showed that the Prx2 is present in the monomeric form at the 22 kilodalton, as you can see the molecular weight mentioned in the slide in the reducing condition, and in the dimeric form at 44 kilodalton under the non-reducing conditions.

These results demonstrated that 4 out of 20 SARS probable cases and 4 of the 20 SARS suspected cases showed higher up-regulation of the Plasma peroxiredoxin 2.

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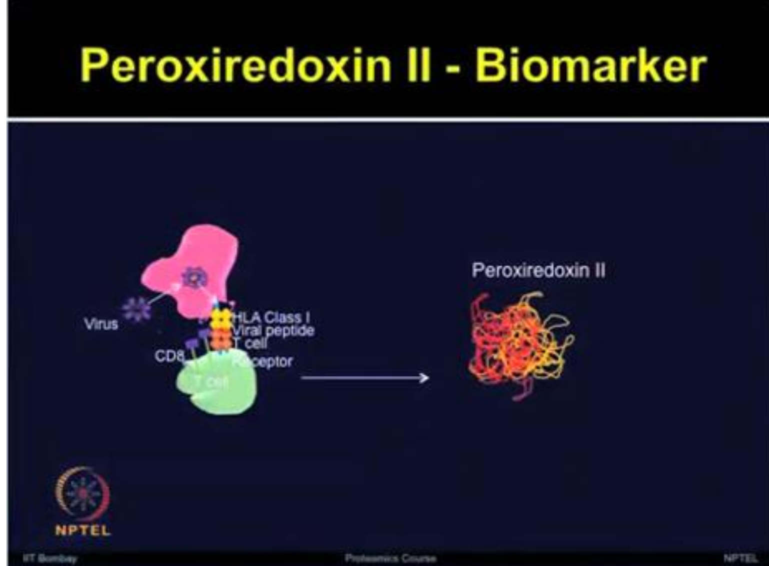

So among various proteins which were interesting, identified from this study peroxiredoxin 2 was appearing quite interesting and also because it was validated by the independent techniques, so the level of Plasma peroxiredoxin 2 in patients with SARS was significantly high. Proteomic analysis and Western blot validation suggested that peroxiredoxin 2 may be used as one of the SARS disease associated biomark.

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

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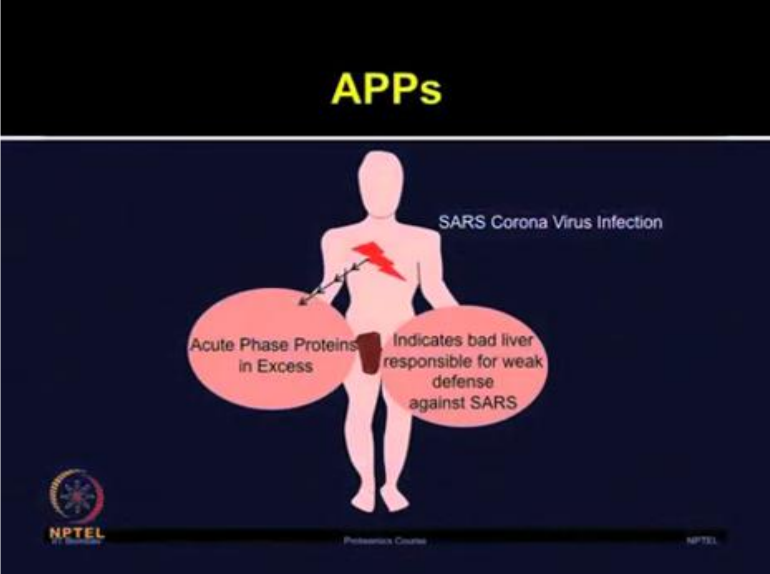

The active innate immune responses and oxidation associated injuries may play major role in the SARS pathogenesis. The Acute-phase proteins found in the SARS patients suggested that

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

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Points to ponder

- High levels of peroxiredoxin II seen in plasma of SARS patients
- PrxII may be used as one of the SARS disease associated biomarkers
- Few acute phase proteins (APP) were also differentially expressed
- Active innate immune responses and oxidation associated injuries may play major role in SARS pathogenesis

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Section II

Challenges associated with Two dimensional gel electrophoresis (2-DE)

Now, in the previous lectures when we talked about obtaining good 2D gel image…

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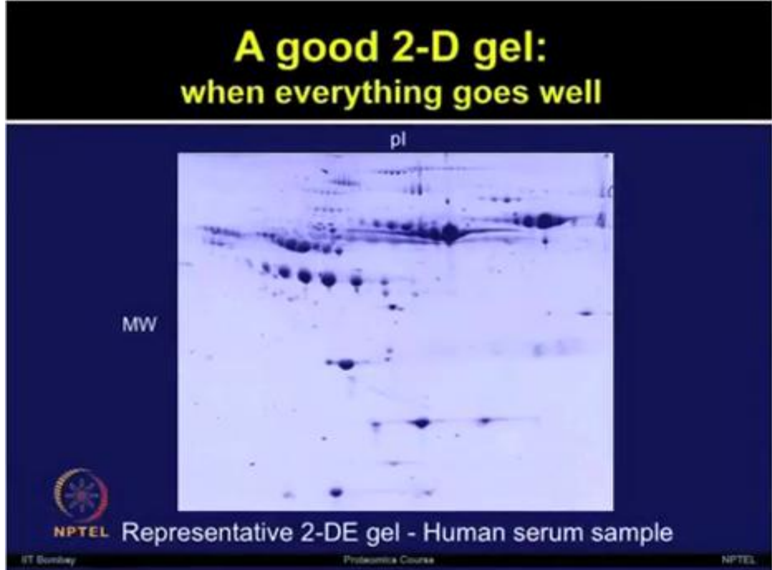

… Then you may realize or feel that at the end of that experiment all the 2D gel image will look very good. So I am showing you one 2D gel which is appearing very good and this is something when everything goes very well. So if your protein separation and staining everything is fine, then at the end 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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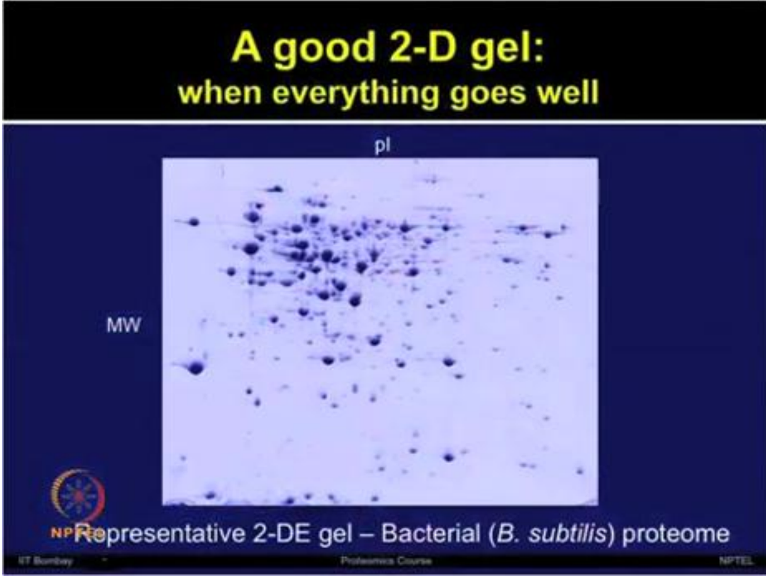

Now I am showing you another good gel obtained from bacterial sample B. subtilis, but often this is not the case.

Many times there are different types of issues which could give rise to very bad images. These issues could be because of the sample preparation method, because of reagents involved, because of isoelectric focusing, different types of parameters involved and finally different types of staining methods being used. So let's talk about some of these issues step by step.

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There are various challenges which are associated with two-dimensional electrophoresis, mainly the gel artifacts are major limitations.

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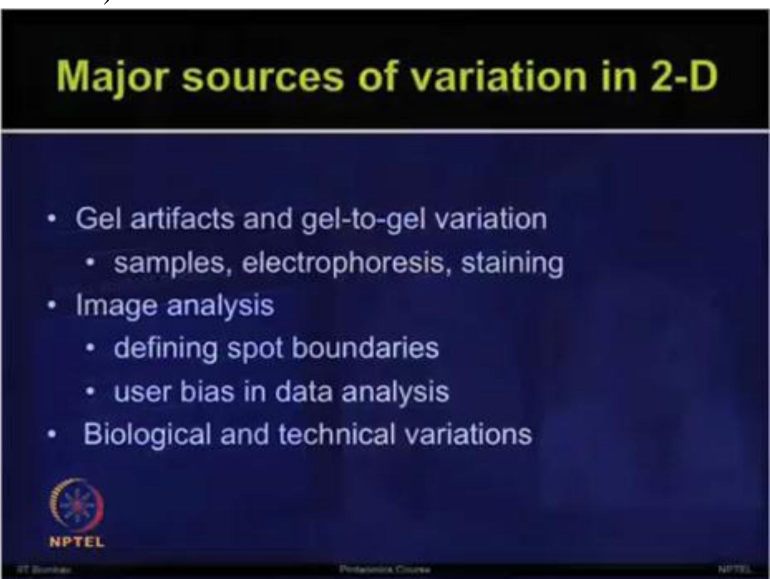

How to compare 2 gels, how to minimize the variations? Often during the electrophoretic run, there is lot of run to run variation. Then you have different types of technical and biological

variations. The image analysis, that itself is 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 are major sources of variation in two-dimensional electrophoresis. I am showing you few problematic gels how even the small mistakes or some issues inherent to your biological samples can give rise to very bad 2D gels.

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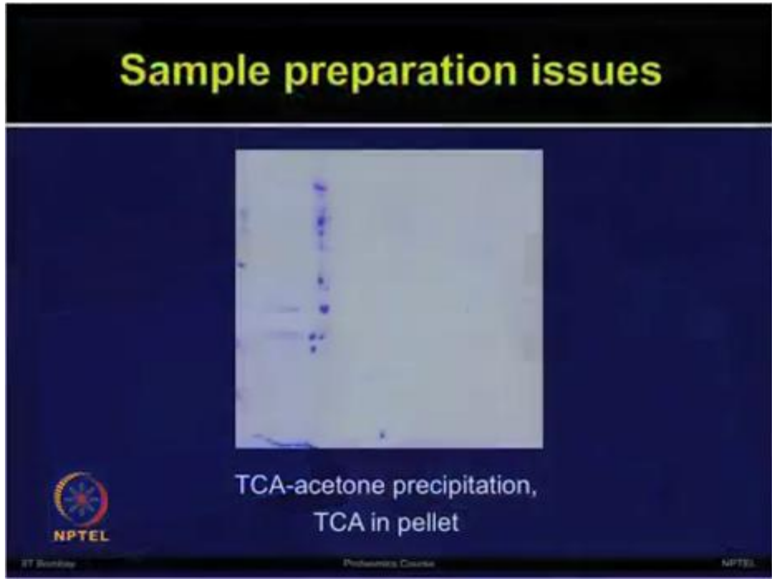

For example I am showing you few sample preparation issues.

In this gel as you can see, the TC 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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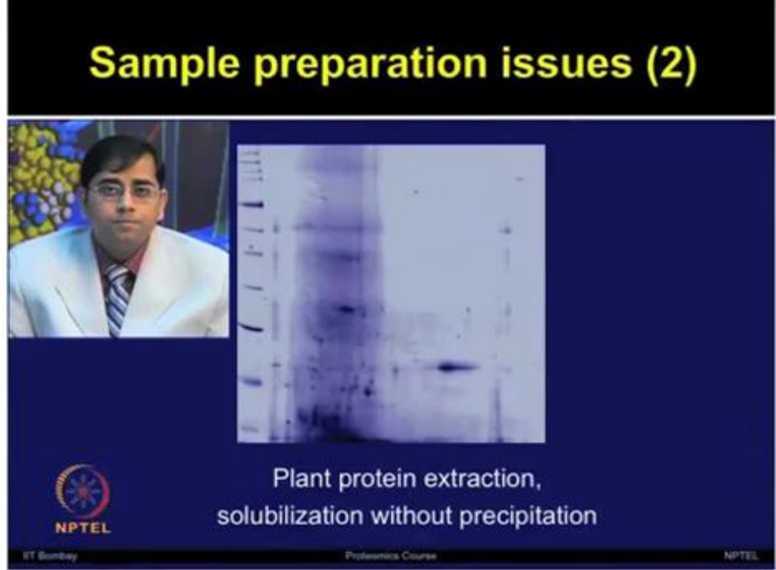

Now if you are performing the plant protein extraction but the solubilization is not sufficient without precipitation, one can see these types of streaking and uneven gel pattern.

Now if you have samples which are having proteins in the abundance such as crude serum samples,

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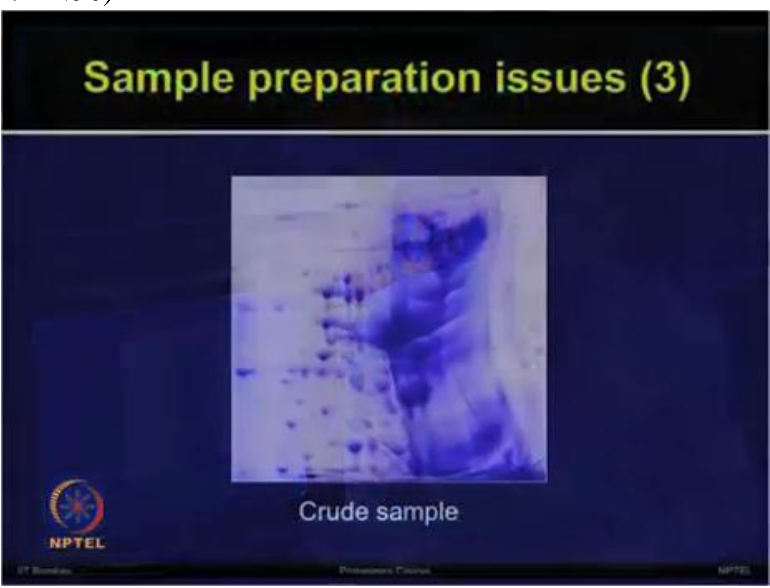

So these samples will show you different interference in the focusing strip because of large salt and other interfering components present and then you can see these types of bad gels because of the salt or different types of abundant proteins present in the mixture.

Now the sample variation is one problem. Often the chemical impurities or the chemical ingredients can also give rise to different types of bad gels…

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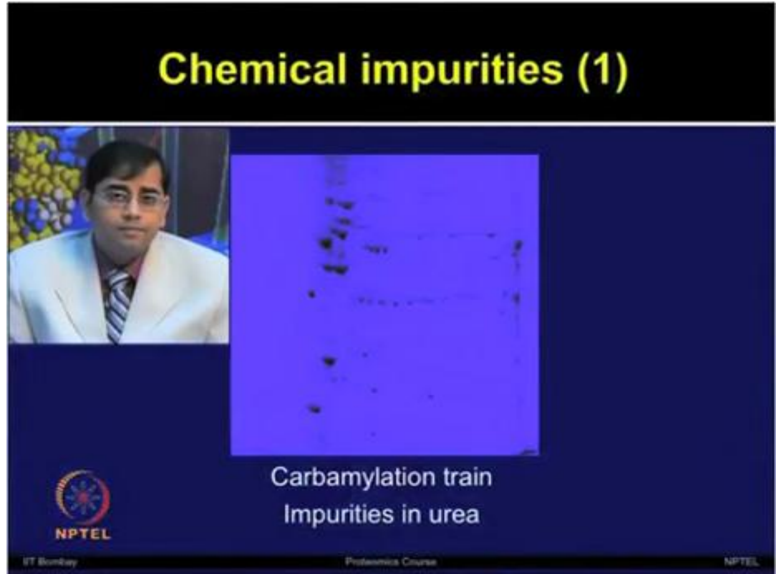

Such as the chemical impurity shown in this gel where the urea is very impure and one can see the carbamylation trail as shown in this gel. If the TEMED is very old... often the TEMED is very...

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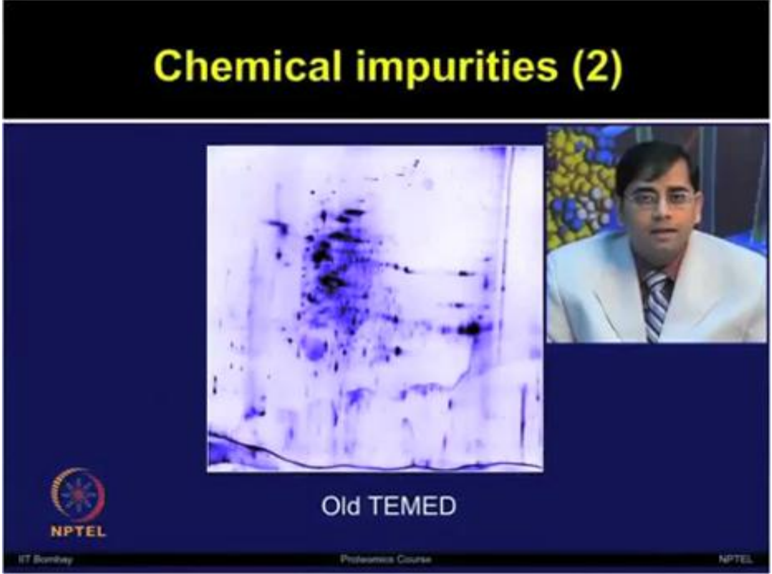

Small amount of TEMED is used in making the second dimension gel SDS PAGE, so people often use that bottle for very long time. And as TEMED is very old, you can see some dark pattern on the gel, something similar to as shown in this image.

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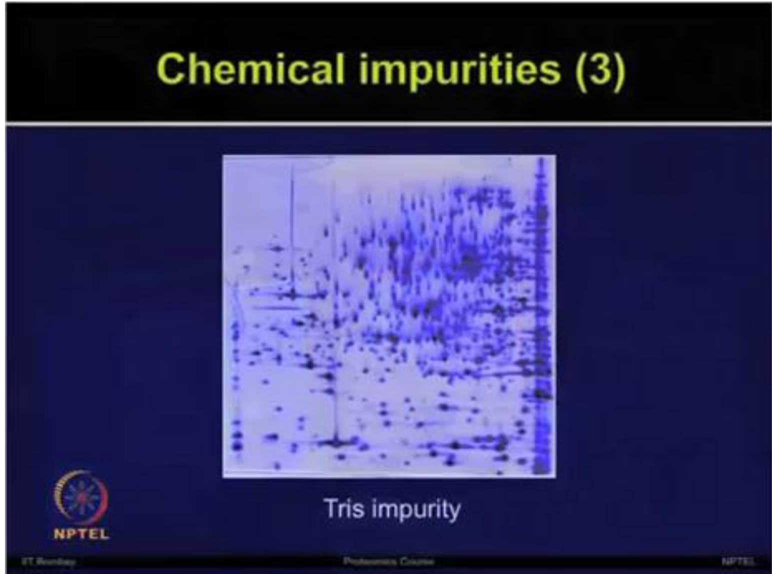

Now if your chemical such as TRIS is not very good quality, it is impure, again that will show some artifacts in the gel.

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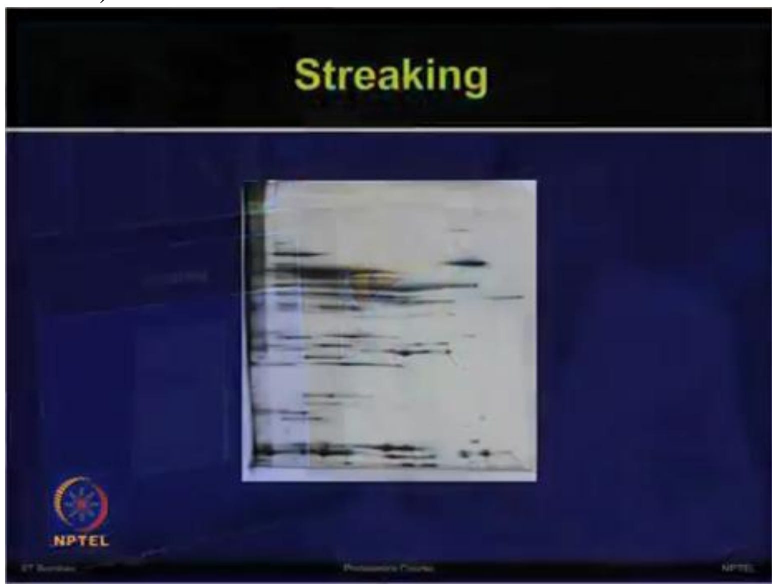

Streaking, that is very oftenly seen because of different types of problems which could be associated....whether it is coming from the sample preparation or because of different types of interfering components.

Often you have nucleic acid or carbohydrates still present in the mixture; the extraction method was not sufficient to eliminate all of those impurities. Many times, salts and other different 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.

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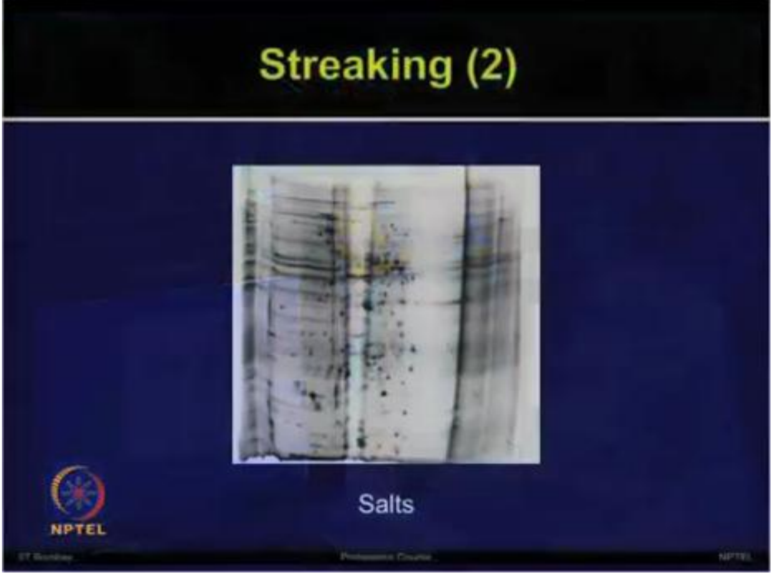

So as shown in this gel, the presence of salt can be interfering and may result into vertical streaking as shown in this gel.

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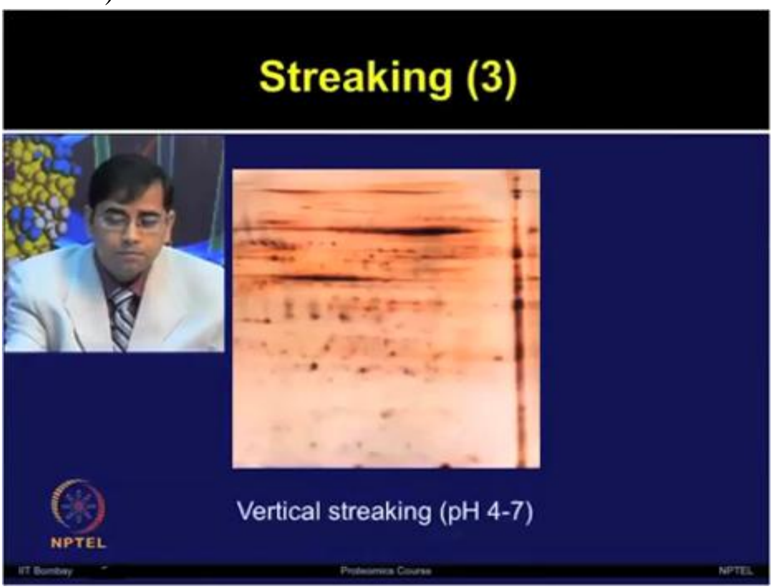

If you are using narrow pH gradient strip such as 4 to 7 pH strip, then the vertical streaking can appear because all the proteins beyond 7 pI will stack together in that region and one can see the vertical streaking.

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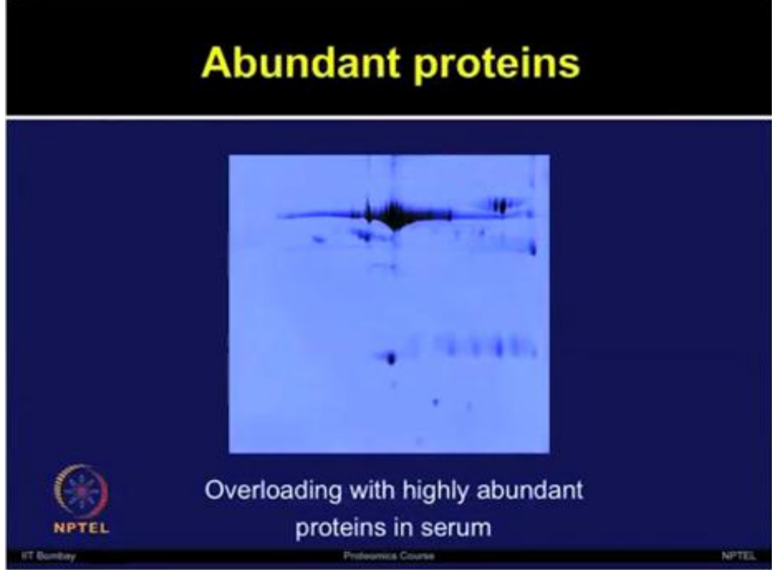

Now abundant proteins, as we briefly talked last time; some impure samples do contain highly abundant proteins and salts. So, abundant proteins are one of the major interfering components which can be easily seen on the gel.

If you have serum sample, remaining interfering components present in the serum including some abundant proteins such as serum albumen...now in the plants one can expect G-BisCO as one of the very large protein present in the plant leaf.

So these abundant proteins, they mask many smaller proteins 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 types of streaking as well as different...the whole region is masked because of the abundance of that protein.

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

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

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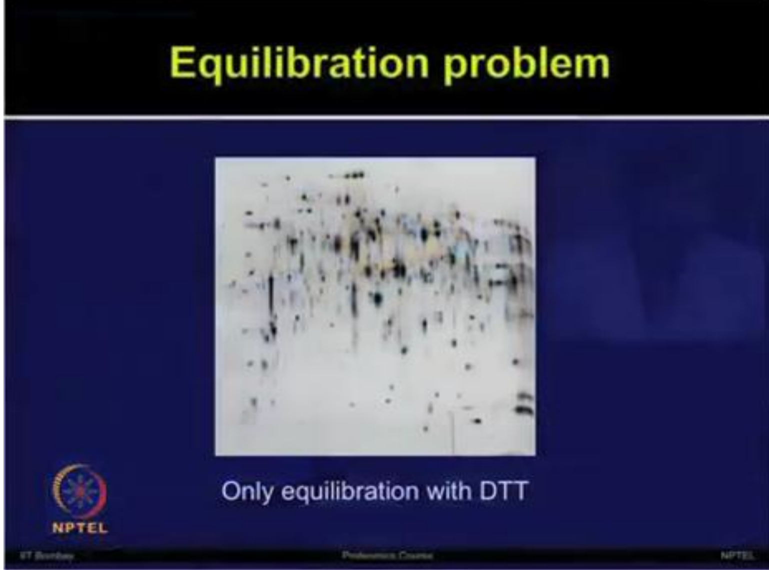

Equilibration process itself can create some problems. For example if you have performed only first equilibration with DTT and forgot to do the second dimension, the second equilibration prior to doing the second dimension separation, then you can see some pattern similar to as shown here, that only equilibration with the DTT.

Many times 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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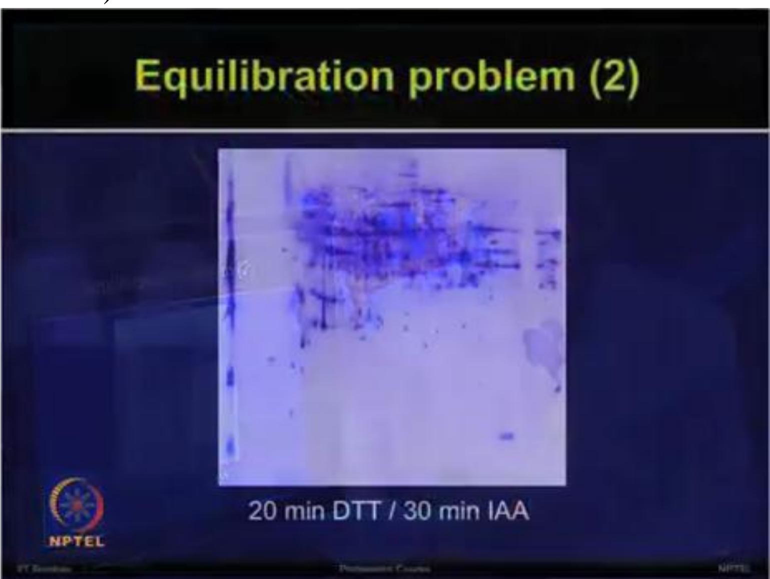

In this gel it is shown that by increasing the equilibration time to 20 minutes of DTT and 30 minutes of iodoacetamide, the gel pattern looks very problematic and that is because of the equilibration excess. Now IEF process is very crucial

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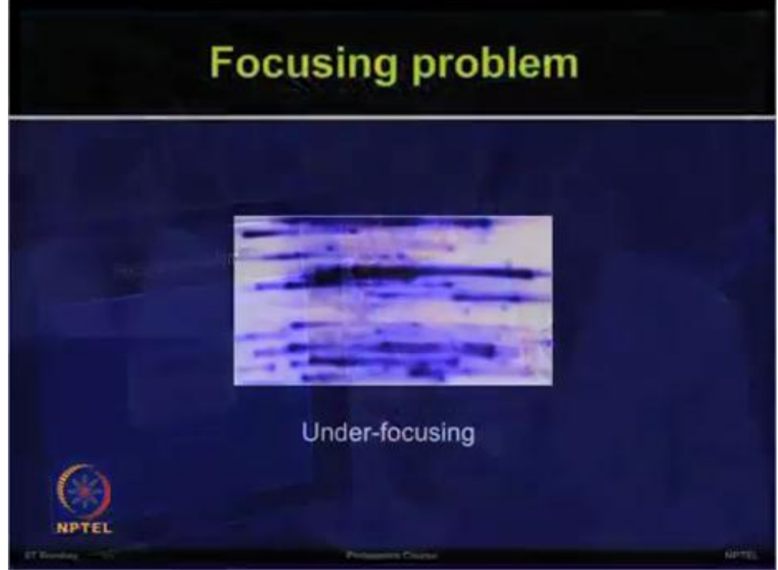

Many times, during the IEF run itself, one can monitor the problems. If you have a software which is showing you how the run is progressing, often if your voltage setting is not correct, insufficient for the complete focusing, it may result into the under-focusing, as shown in the image here for under-focusing.

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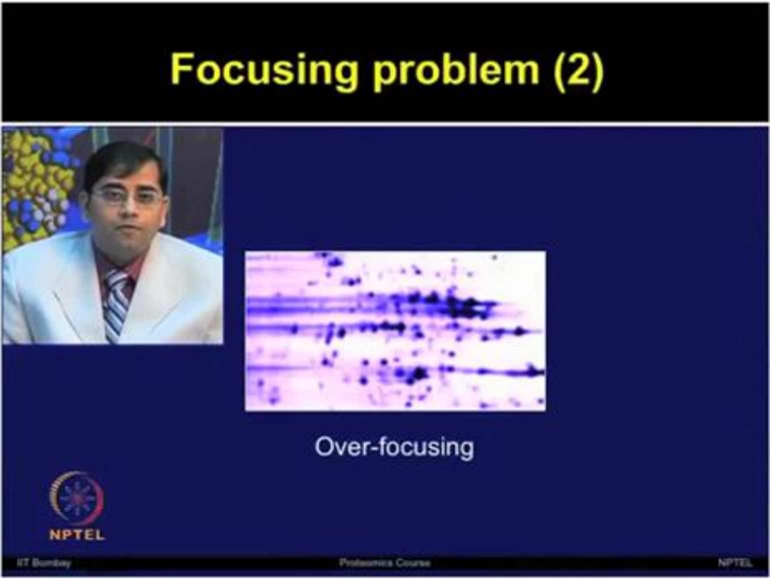

Now if we have run too much voltage and the overall volt-hour is very much, very high, then it may result into over-focusing. So optimizing the focusing protocol for the appropriate duration and the overall volt-hour is very important.

So there are different types of source of variations can be introduced, from the biological as well as technical means.

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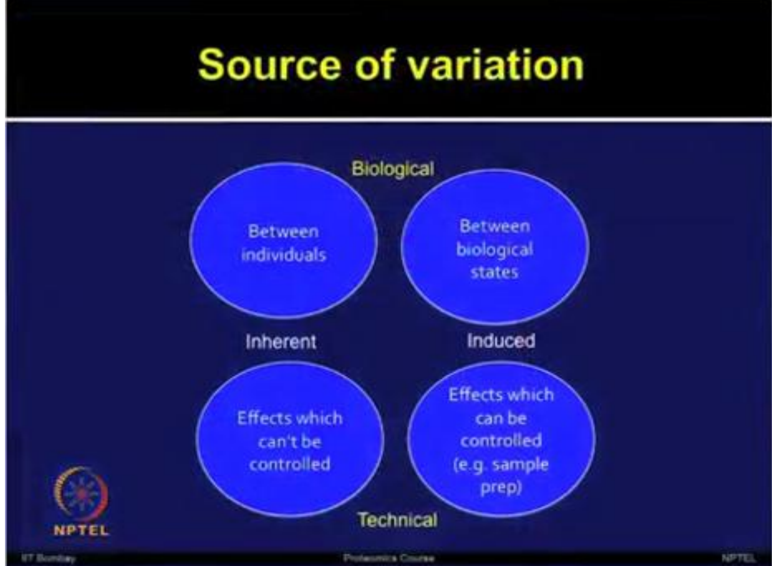

There are different biological variations on which there is no control. If you are doing some clinical studies, then even if you have done proper age and gender match, still there will be some variations due to each individual's uniqueness. So those biological variations are very tough to eliminate.

But then there are lots of technical variations which can be improved, during the sample preparation, during the IEF settings, during the equilibration steps; so all of those steps may result into large variations.

So in two-dimensional electrophoresis, when you are separating your control and treatment gels on 2 different gels and you have so many variations coming from both biological and technical variants, then overall 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 or 2D DIGE have emerged.

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Sources of variation in 2-DE:

- Variations in sample preparation
- IPG strip pH range
- Presence of salt
- Voltage applied for separation
- Equilibration time
- Quality of chemicals used
- User-bias in analysis

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Summary

- Two illustrative case studies:
  - Effect of drug treatment on malaria parasite
  - Plasma proteome analysis of severe acute respiratory syndrome (SARS)
- Challenges associated with 2-DE were discussed