

Capillary-gel-electrophoresis-based- PCR-Ribotyping

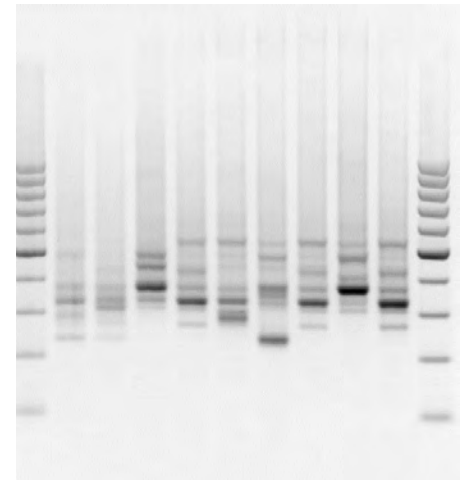
Alexander Indra

ESCMID Online Lecture Library
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Capillary gel electrophoresis based PCR ribotyping

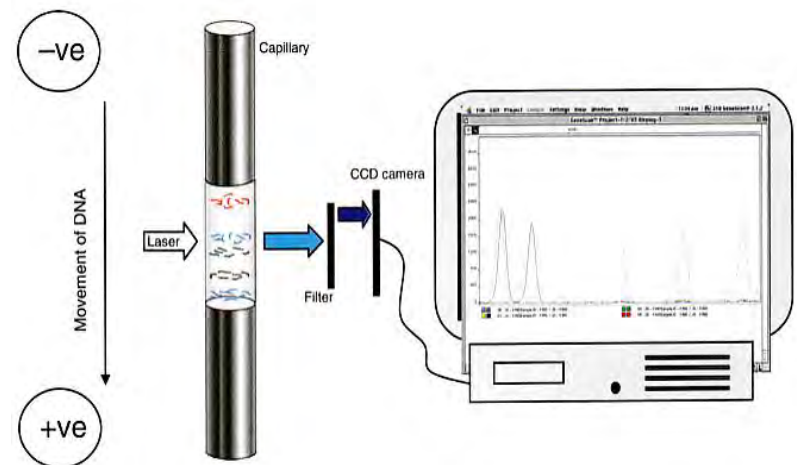
Conventional *Clostridium difficile* PCR-Ribotyping

- problematic inter-laboratory exchange
 - variable use of laboratory specific nomenclature
- no international database
- every sample has to be compared with controls processed under the same conditions
- inconsistent data interpretation of similar band patterns
 - e.g. PCR-ribotype 066
 - known with Toxinotype V and VI
 - untypable 001 subtypes



Capillary gel electrophoresis based PCR ribotyping

- high reproducibility by using
 - a fluorescence labeled primer
 - a uniform size-standards within every sample
 - reproducible running conditions for every sample
- high throughput
- comparability of data results
- easy-to-use data analysis
- higher discriminatory power
 - fragments with 1.5 bases difference or more can be differentiated



Capillary gel electrophoresis based PCR ribotyping

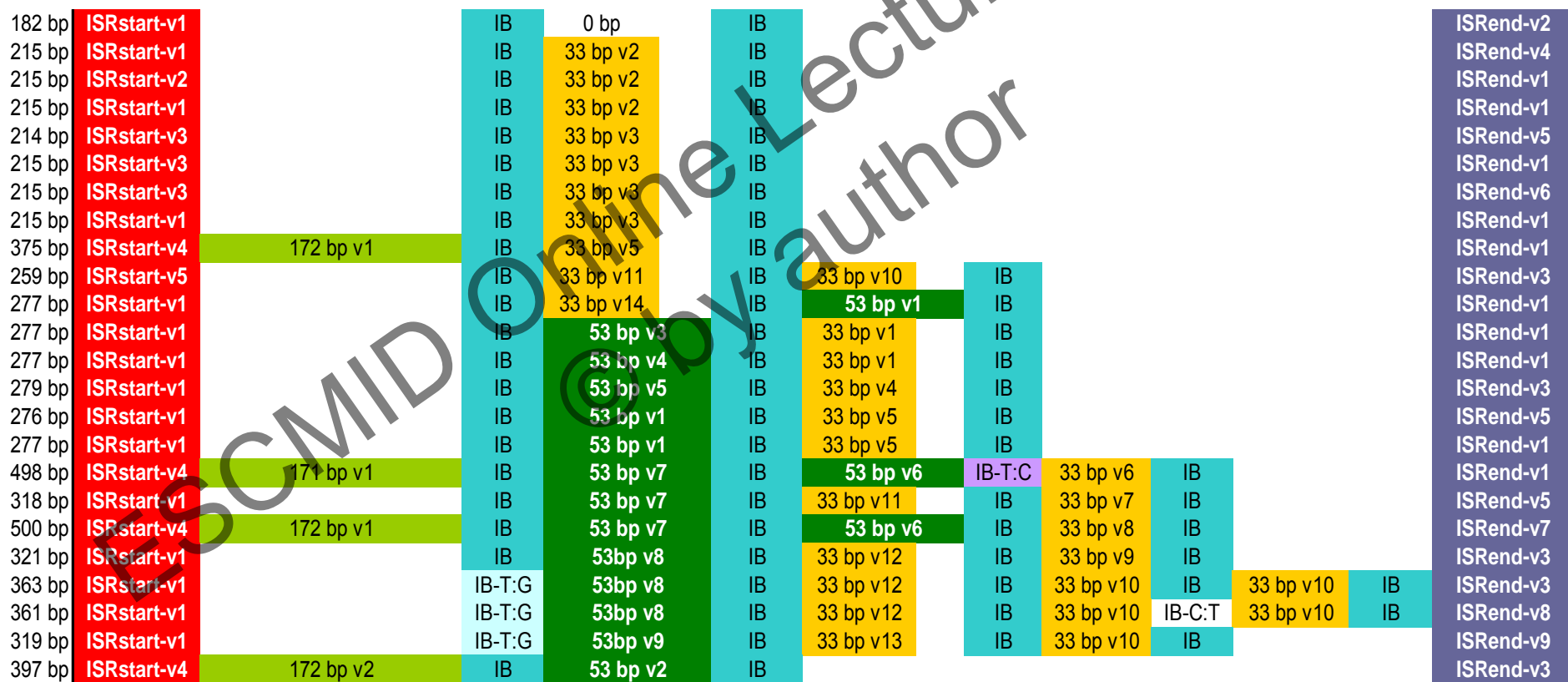
- 6 different 014 subtypes including 020
- Toxinotype V and VI of PCR-ribotype 066 can be differentiated



Mechanisms behind the variations in PCR-Ribotyping patterns

- differences in fragment lengths of the *C. difficile* 16S–23S ISR are based on the number of **9 bp direct repeats** separated by **spacer sequences**
 - spacer sequences detected show **three different lengths**
 - **20 bp**
 - **33 bp**
 - **53 bp**
- some ISR display a 172 bp spacer sequence that includes a gene for tRNA-Ala
- In contrast to the earlier findings by Sadeghifard et al. this results show a **highly structured organization** of the 16S–23S ISR of *C. difficile*

Mechanisms behind the variations in PCR-Ribotyping patterns



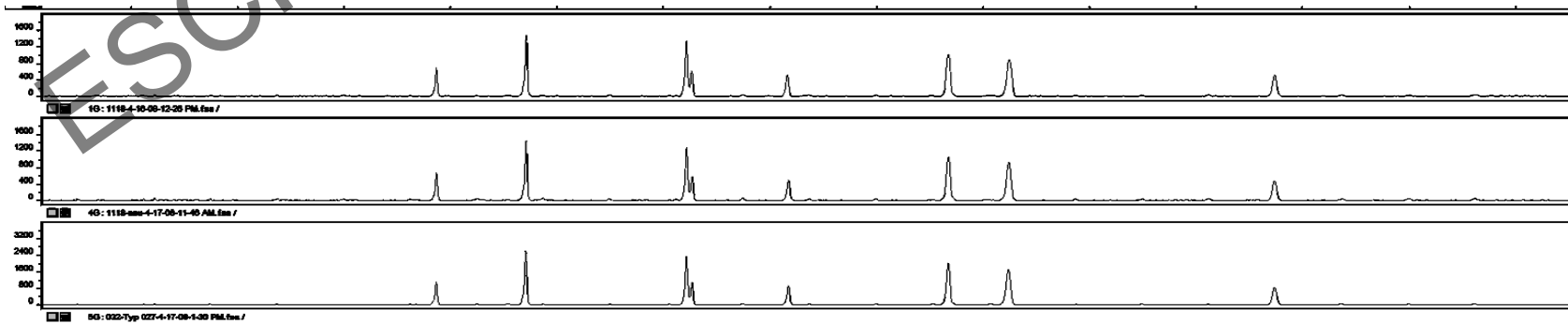
Indra & Blaschitz et al .2010

Mechanisms behind the variations in PCR-Ribotyping patterns

- this composition is responsible for the length variations of the 16S–23S ISR used in PCR ribotyping
- it has evolved as the result of several mechanisms frequently occurring during DNA replication and recombination
 - slipped-strand mispairing
 - intra-chromosomal homologous recombination
 - possible inter-chromosomal homologous recombination

Mechanisms behind the variations in PCR-Ribotyping patterns

- this highly structured and conserved organization is responsible for the comparability and reproducibility found in capillary gel electrophoresis PCR-ribotyping
 - it allows **AGES-WEBRIBO** comparison of results
 - of **different** machines
 - with **different** size-standards
 - with **different** Primer-pairs



LAB-based database Systems: Clostridium difficile

- one laboratory responsible for nomenclature and identification
 - o Laboratory resources are limited
 - o only few had/have access to typing information
- **till now various „localised“ nomenclature systems**
 - o AI, SW,
- limited availability of a standard strain collection
 - o till 2008 !!! no international strain collection available
 - strain collection sample size is limited
 - possibility for errors
 - strain collection is always months or years behind of the users epidemiological development

INTERNET vs LAB based Database Systems



● INTERNET

- **Up to date internet-database for all users**
- **Only** the sequencer **file is needed** to define a new type
- **No additional costs**
 - no soft- or hardware is needed
- **No restriction** for sequencer, gel, size-standard or primer-set
- **ALL users have access** to new Data
- **No shipping** costs

● LOCAL

- Up to date database for **one/few** local users
- **Strain must be send** to reference Lab
- **Additional costs**
 - Soft- or hardware **is needed**
- **Methods restricted** to achieve minimal exchangeability
- **only local User** can access data
- **shipping costs**

WEB-BASED SYSTEMS TO IDENTIFY DNA PROFILES

- available systems

○ HPA-Legionella SBT Typing

Options

Legionella pneumophila Sequence-Based Typing

[Get Sequences](#)

[Compare Profile Strings](#)

Prior to pasting in a query sequence you must ensure that the consensus sequence is in the correct orientation (5'-3') and is trimmed to the correct length. The positions of the target region with respect to the reference sequence are given in "SBT Loci Details" (left), and reference sequences trimmed to the correct position and length can be downloaded to aid contig assembly by clicking "Download Reference Sequences" (left).

Sequence Entry Form

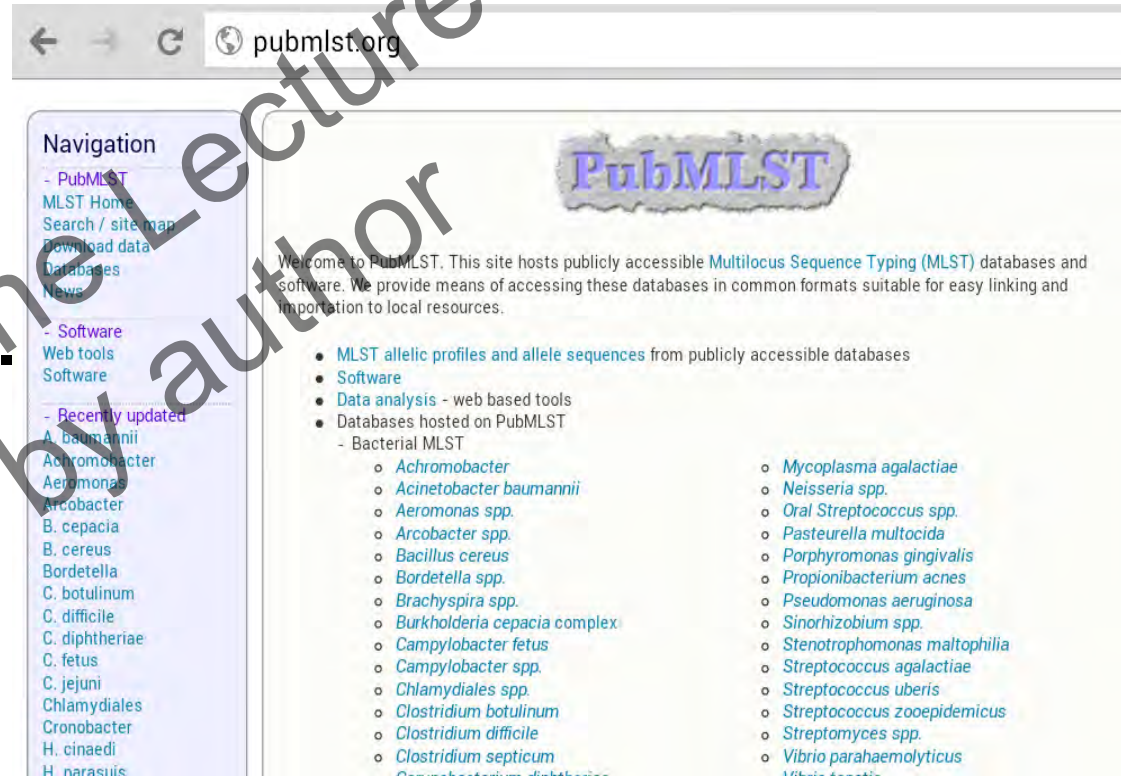
Allele	Sequence	Allele Number	
flaA	<input type="text"/>		<input type="button" value="upload sequence"/>
pilE	<input type="text"/>		<input type="button" value="upload sequence"/>
asd	<input type="text"/>		<input type="button" value="upload sequence"/>
mip	<input type="text"/>		<input type="button" value="upload sequence"/>
mompS	<input type="text"/>		<input type="button" value="upload sequence"/>
proA	<input type="text"/>		<input type="button" value="upload sequence"/>
neuA	<input type="text"/>		<input type="button" value="upload sequence"/>

WEB-BASED SYSTEMS TO IDENTIFY DNA PROFILES

- available systems

○ HPA-Legionella Typing

○ **various MLST Databases**



The screenshot shows the PubMLST website interface. The browser address bar displays 'pubmlst.org'. The page features a 'Navigation' sidebar on the left with links for 'PubMLST', 'MLST Home', 'Search / site map', 'Download data', 'Databases', and 'News'. Below this, there are sections for 'Software' (Web tools, Software) and 'Recently updated' (listing various bacterial species like A. baumannii, Achromobacter, Aeromonas, etc.). The main content area includes a 'PubMLST' logo and a welcome message: 'Welcome to PubMLST. This site hosts publicly accessible Multilocus Sequence Typing (MLST) databases and software. We provide means of accessing these databases in common formats suitable for easy linking and importation to local resources.' Below the welcome message is a list of resources:

- MLST allelic profiles and allele sequences from publicly accessible databases
- Software
- Data analysis - web based tools
- Databases hosted on PubMLST
 - Bacterial MLST
 - Achromobacter
 - Acinetobacter baumannii
 - Aeromonas spp.
 - Arcobacter spp.
 - Bacillus cereus
 - Bordetella spp.
 - Brachyspira spp.
 - Burkholderia cepacia complex
 - Campylobacter fetus
 - Campylobacter spp.
 - Chlamydiales spp.
 - Clostridium botulinum
 - Clostridium difficile
 - Clostridium septicum
 - Mycoplasma agalactiae
 - Neisseria spp.
 - Oral Streptococcus spp.
 - Pasteurella multocida
 - Porphyromonas gingivalis
 - Propionibacterium acnes
 - Pseudomonas aeruginosa
 - Sinorhizobium spp.
 - Stenotrophomonas maltophilia
 - Streptococcus agalactiae
 - Streptococcus uberis
 - Streptococcus zooepidemicus
 - Streptomyces spp.
 - Vibrio parahaemolyticus

WEB-BASED SYSTEMS TO IDENTIFY DNA PROFILES

- available systems

- HPA-Legionella SBT Typing

- various MLST-Databases

- **MIRU-VNTRplus**



The screenshot shows the MIRU-VNTRplus web application interface. The browser address bar displays www.miru-vntrplus.org/MIRU/index.far. The page title is "MIRU-VNTRplus". A navigation menu on the left includes: Home, Browse Database, Identification by Similarity Search, Nomenclature, Background, Policy, Help, About us, Contact us, and Imprint. The main content area features a welcome message: "Welcome to the MIRU-VNTRplus web application!". Below this, there is a link to "New to MIRU-VNTRplus? Click here to open an example data set!". A section titled "Use the Home-button to return to this page at any time, or select 'Home' from the Navigate-menu." is followed by a prompt: "Select one of the commands below to work with the database:". Underneath, it states "You have uploaded 0 user strains." and provides two buttons: "Enter a single user strain" and "Import multiple strains from file or clipboard". A "Change Settings" section includes buttons for "Change VNTR loci set" (set to 24 loci), "Change VNTR loci order", and "Reset default parameters". At the bottom, there is a paragraph of text and a circular image showing a DNA microarray or gel electrophoresis pattern.

Molecular typing of bacteria from the *Mycobacterium tuberculosis* complex (MTBC) is essential for epidemiological purposes such as investigating the spreading of specific genotypes. Recently, mycobacterial interspersed repetitive units (MIRU) typing has become an important method, as it allows high-throughput, discriminatory and reproducible analysis of clinical isolates. MIRU is a MTBC specific name of a multiple locus VNTR (variable number of tandem repeats) analysis (MLVA) bacterial typing scheme. Because of its portable data format, MIRU typing has the potential to be a versatile tool for individual strain identification based on large reference databases. However, specialized bioinformatic web tools to analyze MIRU data and public reference databases are not available.

To meet this need, a collection of 186 strains representing the major MTBC lineages was used for implementing a web server. MIRU-VNTRplus (<http://www.miru-vntrplus.org/>). For each strain species, lineage, and epidemiologic information was stored together with copy numbers of 24 MIRU loci, spoligotyping patterns, regions of difference (RD) profiles, single nucleotide polymorphisms (SNPs), susceptibility data, and IS6110 RFLP fingerprint images.

WEB-BASED SYSTEMS TO IDENTIFY DNA PROFILES

- available systems

○ HPA-Legionella SBT Typing

○ various MLST-Databases

○ MIRU-VNTRplus

○ **RIDOM-StaphType**

The screenshot shows the 'Ridom SpaServer - Submit a new spa-type' page. It features a navigation menu on the left with options like Overview, Database, and Contact. The main content area contains a note about submission requirements, a table of signature sequences, and a form for entering contact and strain data.

Note: Ridom StaphType users are requested to submit their new spa-repeats and -types via the StaphType software synchronization process. This form is intended only for SpaServer users not (yet :-)) using the software. Please note, that we only accept NEW repeats and types!

The sequences must contain the following 5' and 3' signatures on the correct positions to allow a reliable spa-typing:

	Signature Sequence	Distance to Repeats	Regions
5' Signature	RCA MCA AAA	0	1156-1164; -9 to -1
3' Signature	TAY ATG TCG T	19 or 18	1472-1481; -19 to +28 or +20 to -29

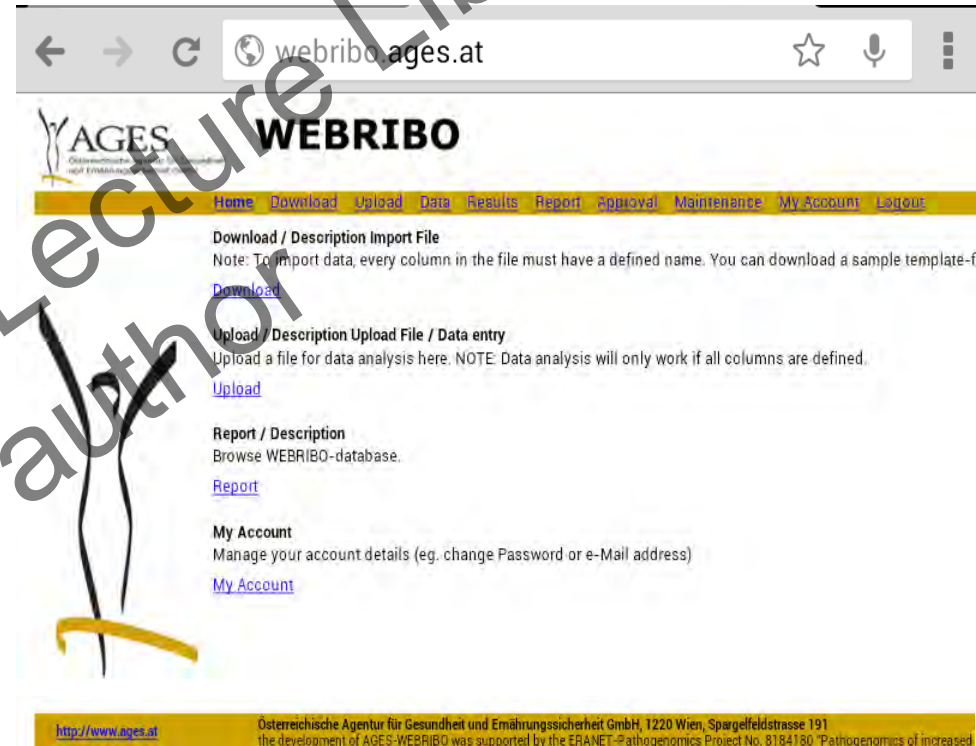
The signatures are numbered according to the forward strand of *S. aureus* (GenBank accession no. J01786).

Contact Data		Strain Data	
Salutation:	Mr. ▼	Isolate ID:	<input type="text"/>
Title:	<input type="text"/>	Isolation Date:	▼ ▼ ▼
First name:	<input type="text"/>	Chromatogram Files (in SCF or ABI format)	
Last name:	<input type="text"/>	Forward File:	Datei auswählen Keine ausgewählt
Email:	<input type="text"/>	Reverse File:	Datei auswählen Keine ausgewählt
Organisation:	<input type="text"/>	Country:	▼ *
Department:	<input type="text"/>	State:	<input type="text"/>
Street:	<input type="text"/>	City:	<input type="text"/>
Street number:	<input type="text"/>	Zip:	<input type="text"/>
ZIP:	<input type="text"/>	MRSA / MSSA:	▼ *
City:	<input type="text"/>	MLST-type:	<input type="text"/>
State:	<input type="text"/>		
Country:	▼ *		

WEB-BASED SYSTEMS TO IDENTIFY DNA PROFILES

- available systems

- HPA-Legionella SBT Typing
- various MLST-Databases
- MIRU-VNTRplus
- RIDOM-StaphType
- **AGES-WEBRIBO**



The screenshot shows the AGES WEBRIBO website. The browser address bar displays 'webribo.ages.at'. The website header includes the AGES logo and the text 'WEBRIBO'. A navigation menu contains links for Home, Download, Upload, Data, Results, Report, Approval, Maintenance, My System, and Logout. The main content area is divided into three sections: 'Download / Description Import File' with a note about file naming and a 'Download' link; 'Upload / Description Upload File / Data entry' with a note about column definitions and an 'Upload' link; and 'Report / Description' with a 'Report' link. A 'My Account' section offers to manage account details with a 'My Account' link. The footer contains the website URL 'http://www.ages.at' and information about the Austrian Agency for Health and Food Safety (AGES) and the ERANET-Pathogenomics project.

the only internet database doing automated fragment analysis

WEBRIBO

webribo.ages.at

- WEBRIBO allows comparison of results
 - on **different** machines
 - **different** capillaries
 - **different** POP-Gels
 - with **different** size-standards
 - with **different** Primer-pairs (Bidet, Stubbs, Janežič)

Capillary Gel Electrophoresis based PCR Ribotyping Analysis using WEBRIBO

Labwork

- Culture
- DNA-Extraction
- PCR
- Capillary PCR-ribotyping

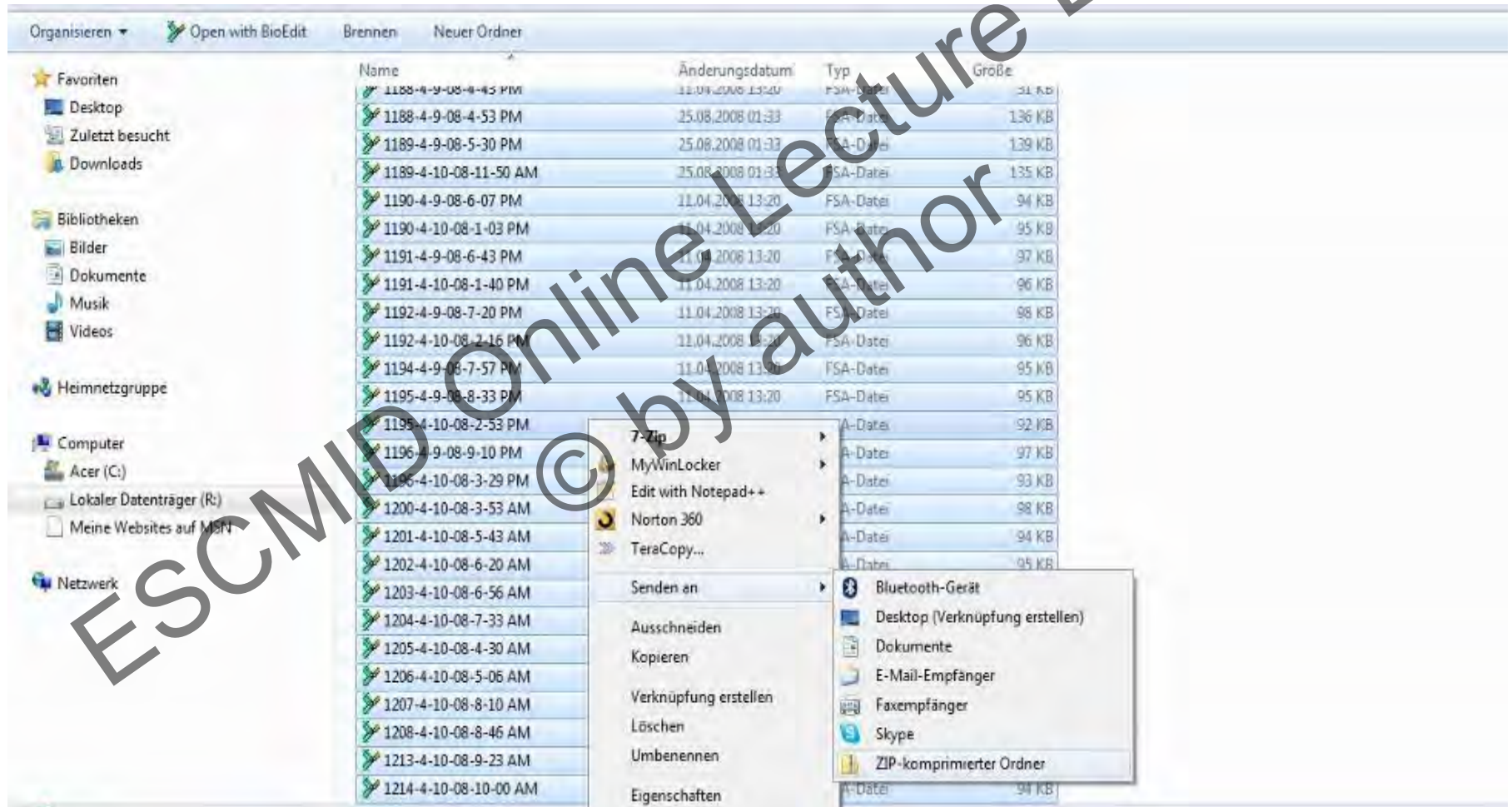
Analysis

- results in 4 steps using **WEBRIBO** (webribo.ages.at)



Applied Biosystems fsa-File Analysis

Step 1: ZIP it



Applied Biosystems fsa-File Analysis

Step 2: Log into WEBRIBO

AGES
Gefährdungsagentur für Gesundheit
und Ernährungssicherheit (AGES)

WEBRIBO

[Home](#)

Welcome!
WEBRIBO allows you to automatically analyse and compare your *Clostridium difficile* capillary-sequencer-based PCR-ribotyping-data and simplifies laboratory PCR-ribotyping methods.

Email:

Password:

[Forgot your password?](#)
[Create user account](#)
[Help](#)

Applied Biosystems fsa-File Analysis

Step 3: Upload your files

webribo.ages.at/index.php?module=upload&action=show

AGES
Genomische Analyse für Clinischen
und Ernährungswissenschaftler

WEBRIBO

Home Download **Upload** Data Results Reports My Account Logout

Upload Section
First select the country of origin (where the strains come from), NOTE: If none is selected, the pre-set country is used. Then, click "Browse" and select an MS Excel-file or a CSV-file for upload.

Country:* Austria

Settings ABI 3130 Series Liz 1200 Pop 7 35 cm 60° C Bidet

Data File:* ABI 310 ABI 3130 Series ABI 3500 Series ABI 3100 Series ABI 3700 Series Beckman Test

Durchsuchen...

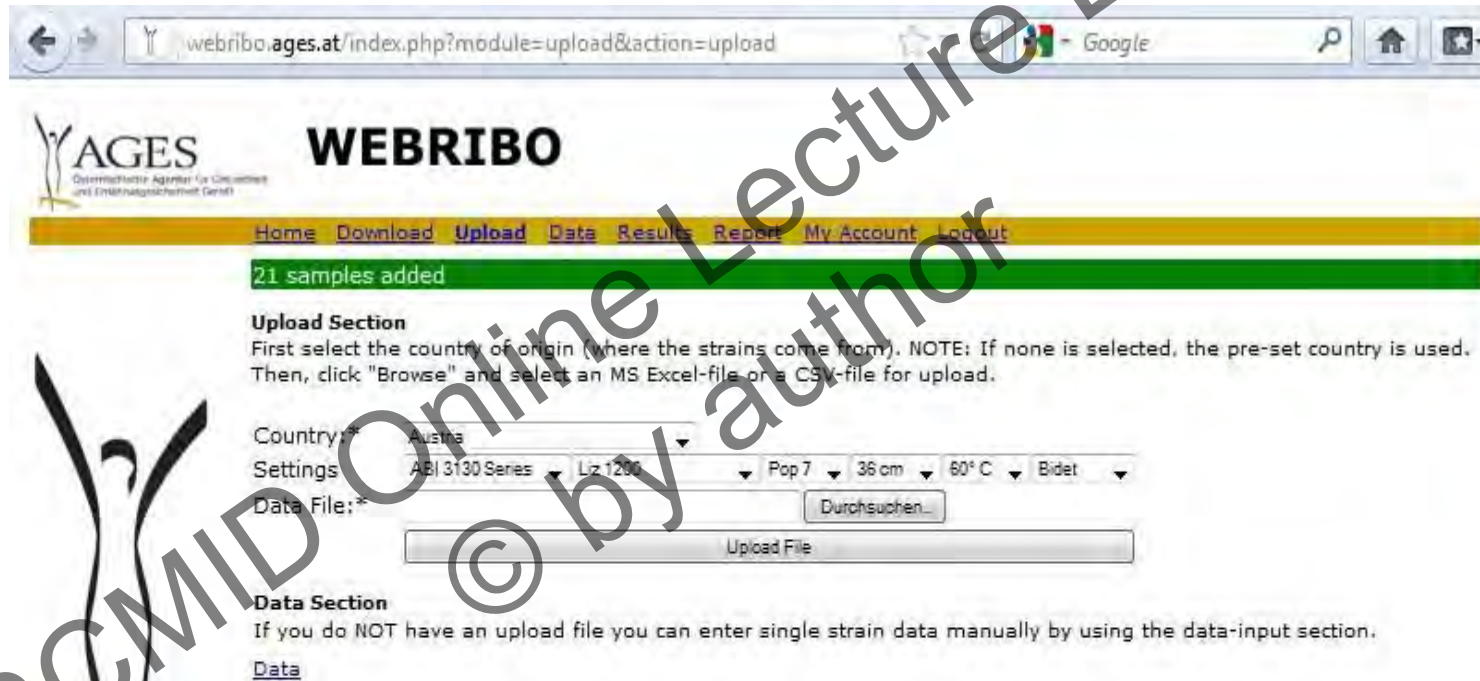
Upload File

Data Section
If you do NOT file you can enter single strain data manually by using the data-input section.

Data

Applied Biosystems fsa-File Analysis

Step 3: Upload your files



The screenshot shows the WEBRIBO web interface. The browser address bar displays the URL: `webribo.ages.at/index.php?module=upload&action=upload`. The page header includes the AGES logo (Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH) and the title "WEBRIBO". A navigation menu contains links for Home, Download, Upload, Data, Results, Report, My Account, and Logout. A green banner indicates "21 samples added".

Upload Section
First select the country of origin (where the strains come from). NOTE: If none is selected, the pre-set country is used. Then, click "Browse" and select an MS Excel-file or a CSV-file for upload.

Country:* Austria
Settings: ABI 3130 Series Liz 1200 Pop 7 36 cm 60° C Bidet
Data File:*

Data Section
If you do NOT have an upload file you can enter single strain data manually by using the data-input section.
[Data](#)

Applied Biosystems fsa-File Analysis

Step 4: Analysis

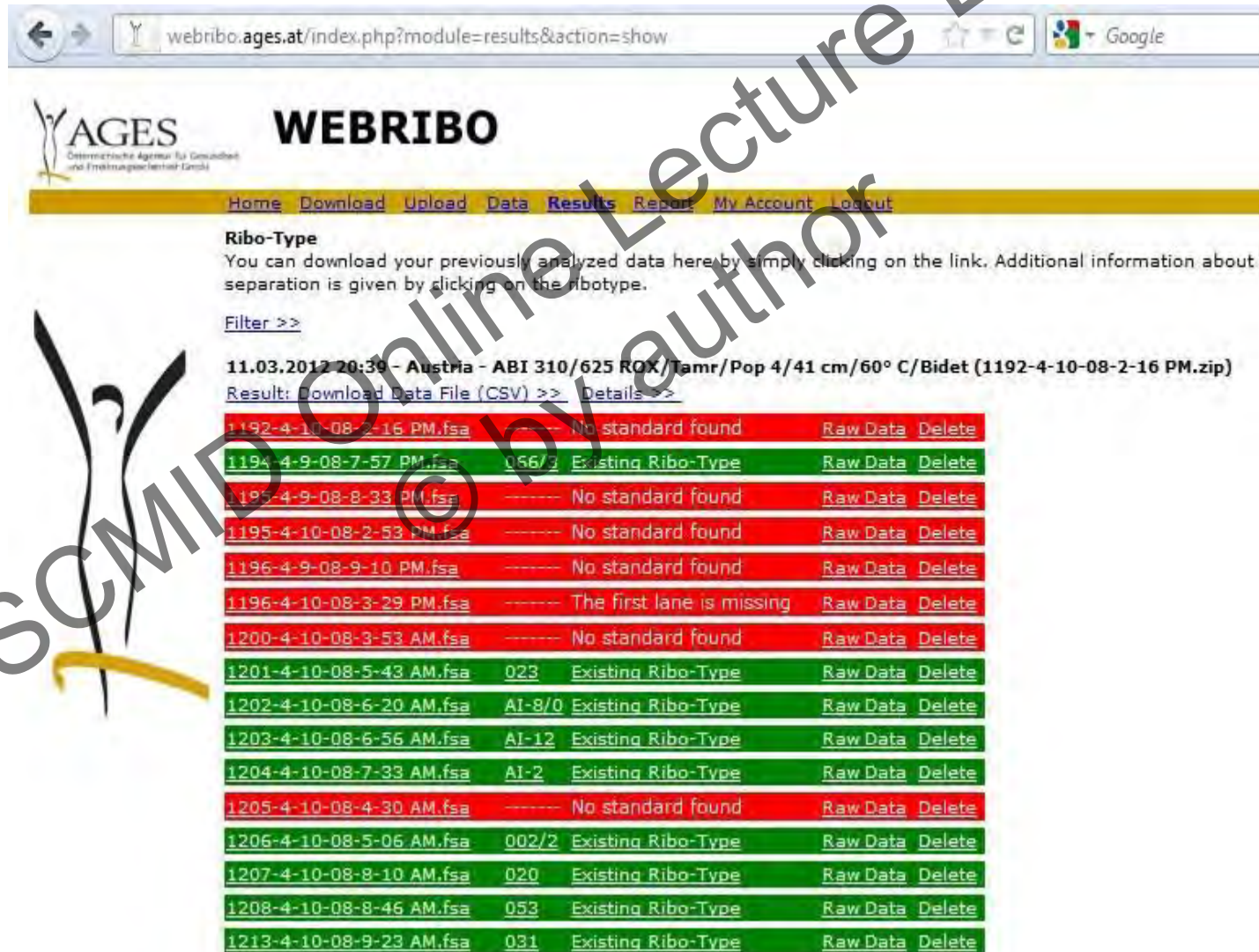


The screenshot shows the WEBRIBO web interface. The browser address bar displays the URL: `webribo.ages.at/index.php?module=results&action=show`. The page header includes the AGES logo (Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH) and the title "WEBRIBO". A navigation menu contains links for Home, Download, Upload, Data, Results, Report, My Account, and Logout. The main content area is titled "Ribo-Type" and provides instructions on downloading data. Below this, there is a "Filter >>" link and a specific analysis entry: "11.03.2012 20:39 - Austria - ABI 3130 Series / Liz 1200 / Pop 7/36 cm/60° C/Bidet (1192-4-10-08-2-16 PM.zip)". Underneath this entry, there are links for "Result: Download Data File (CSV) >>" and "Details >>". The core of the page is a table listing individual fsa files, each with a status of "pending...", a "Raw Data" link, and "Process" and "Delete" buttons.

File Name	Status	Raw Data	Process	Delete
1192-4-10-08-2-16 PM.fsa	pending...	Raw Data	Process	Delete
1194-4-9-08-7-57 PM.fsa	pending...	Raw Data	Process	Delete
1195-4-9-08-8-33 PM.fsa	pending...	Raw Data	Process	Delete
1195-4-10-08-2-53 PM.fsa	pending...	Raw Data	Process	Delete
1196-4-9-08-9-10 PM.fsa	pending...	Raw Data	Process	Delete
1196-4-10-08-3-29 PM.fsa	pending...	Raw Data	Process	Delete
1200-4-10-08-3-53 AM.fsa	pending...	Raw Data	Process	Delete
1201-4-10-08-5-43 AM.fsa	pending...	Raw Data	Process	Delete
1202-4-10-08-6-20 AM.fsa	pending...	Raw Data	Process	Delete
1203-4-10-08-6-56 AM.fsa	pending...	Raw Data	Process	Delete
1204-4-10-08-7-33 AM.fsa	pending...	Raw Data	Process	Delete
1205-4-10-08-4-30 AM.fsa	pending...	Raw Data	Process	Delete
1206-4-10-08-5-06 AM.fsa	pending...	Raw Data	Process	Delete
1207-4-10-08-8-10 AM.fsa	pending...	Raw Data	Process	Delete
1208-4-10-08-8-46 AM.fsa	pending...	Raw Data	Process	Delete

Applied Biosystems fsa-File Analysis

Step 4: Analysis



AGES Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH

WEBRIBO

Home Download Upload Data Results Report My Account Logout

Ribo-Type
You can download your previously analyzed data here by simply clicking on the link. Additional information about separation is given by clicking on the ribotype.

[Filter >>](#)

11.03.2012 20:39 - Austria - ABI 310/625 ROX/Tamr/Pop 4/41 cm/60° C/Bidet (1192-4-10-08-2-16 PM.zip)
Result: [Download Data File \(CSV\)](#) >> [Details](#) >>

1192-4-10-08-2-16 PM.fsa	-----	No standard found	Raw Data	Delete
1194-4-9-08-7-57 PM.fsa	056/3	Existing Ribo-Type	Raw Data	Delete
1195-4-9-08-8-33 PM.fsa	-----	No standard found	Raw Data	Delete
1195-4-10-08-2-53 PM.fsa	-----	No standard found	Raw Data	Delete
1196-4-9-08-9-10 PM.fsa	-----	No standard found	Raw Data	Delete
1196-4-10-08-3-29 PM.fsa	-----	The first lane is missing	Raw Data	Delete
1200-4-10-08-3-53 AM.fsa	-----	No standard found	Raw Data	Delete
1201-4-10-08-5-43 AM.fsa	023	Existing Ribo-Type	Raw Data	Delete
1202-4-10-08-6-20 AM.fsa	A1-8/0	Existing Ribo-Type	Raw Data	Delete
1203-4-10-08-6-56 AM.fsa	A1-12	Existing Ribo-Type	Raw Data	Delete
1204-4-10-08-7-33 AM.fsa	A1-2	Existing Ribo-Type	Raw Data	Delete
1205-4-10-08-4-30 AM.fsa	-----	No standard found	Raw Data	Delete
1206-4-10-08-5-06 AM.fsa	002/2	Existing Ribo-Type	Raw Data	Delete
1207-4-10-08-8-10 AM.fsa	020	Existing Ribo-Type	Raw Data	Delete
1208-4-10-08-8-46 AM.fsa	053	Existing Ribo-Type	Raw Data	Delete
1213-4-10-08-9-23 AM.fsa	031	Existing Ribo-Type	Raw Data	Delete

Applied Biosystems fsa-File Analysis

Compare your results



What is a PR-Type?

- PR Values are assigned

- For new ribotypes
- Ribotype patterns similar to ones in the database but gained with different sequencers, standards, capillaries (BM Values < 3)

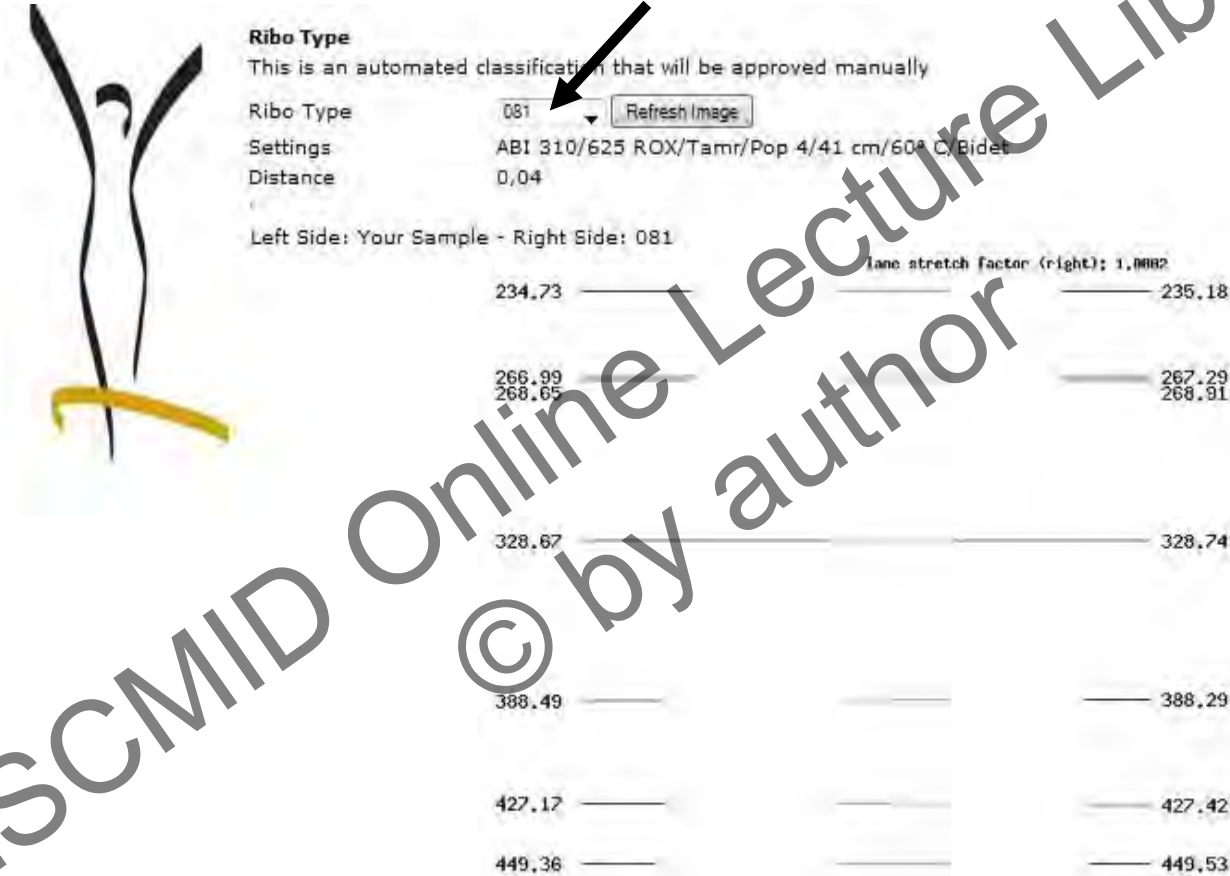
- "most likely" relation to ribotype is given

- comparison can be done by clicking on the link

[Result: Download Data File \(CSV\) >>](#) [Details >>](#)

1177-4-9-08-9-46 PM.fsa	PR02463	Most Likely: 081	Raw Data	Delete
1178-4-9-08-10-23 PM.fsa	PR02463	Most Likely: 081	Raw Data	Delete
1180---4-9-08-11-00 PM.fsa	PR02464	Most Likely: AI-56	Raw Data	Delete
1181-4-9-08-11-36 PM.fsa	PR02465	New Ribo-Type	Raw Data	Delete
1182-4-10-08-12-13 AM.fsa	PR02466	New Ribo-Type	Raw Data	Delete

What is a PR-Type?



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What is a PR-Type?



Ribo Type
Settings
Distance

078 Refresh Image

ABI 310/625 ROX/Tamr/Pop 4/41 cm/60° C/Bidet
3.250,85

Left Side: Your Sample - Right Side: 078

lane stretch factor (right): 1,0875



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What is a PR-Type?



- PR Values are assigned

- For new ribotypes
- Ribotype patterns similar to ones in the database but gained with different sequencers, standards, capillaries (BM Values <3)
 - "most likely" relation to ribotype is given
 - comparison can be done by clicking on the link
 - the final assignment is currently done manually
 - automatic assignment in next update (April-May 2012)

[Result: Download Data File \(CSV\) >>](#) [Details >>](#)

1177-4-9-08-9-46 PM.fsa	PR02463	Most Likely: 081	Raw Data	Delete
1178-4-9-08-10-23 PM.fsa	PR02463	Most Likely: 081	Raw Data	Delete
1180---4-9-08-11-00 PM.fsa	PR02464	Most Likely: AI-56	Raw Data	Delete
1181-4-9-08-11-36 PM.fsa	PR02465	New Ribo-Type	Raw Data	Delete
1182-4-10-08-12-13 AM.fsa	PR02466	New Ribo-Type	Raw Data	Delete

WEBRIBO:

Equipment requirements

● Sequencer

- Applied Biosystems (ABI) Systems tested (310, 3100, 3130(xl), 3730)
 - any POP-Gel
 - any capillary length
- Beckman Coulter Sequencer

● Primers

- any PCR-Ribotyping Primer available (e.g. Bidet, Stubbs or **Janežič**)

● Size-Standard

- any Size-Standard like
 - Geneflo 625 ROX/Tamra
 - LIZ 600
 - LIZ 1200
 - Genemapper 1000
 - Beckman GenomeLab 600

WEBRIBO:

Software requirements

- Computer
 - PC, Mac, Linux,...
 - iPhone, Android, Amazon Kindle....
- Webbrowser
 - IE, Safari, Chrome, Opera,....
- Raw-Data Analysis Software
 - ABI-Peakscanner-Pro (free Software)
 - Beckman
- Spreadsheet Software
 - LibreOffice, Excel, OpenOffice, KDE-Office,

WEBRIBO

webribo.ages.at

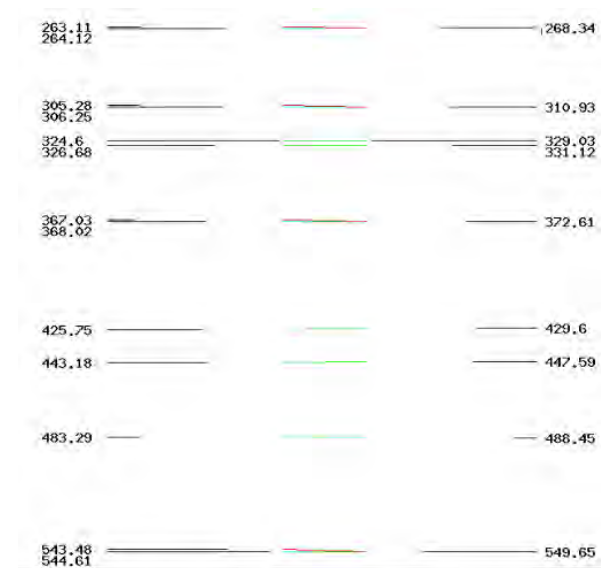
- Up to 99% correct identification of samples analysed with

- **different** size-standards
- **different** sequencing machines
- **different** primers

○ Stubbs, Bidet, Janežič

- a **supervised learning algorithm**

copers with double-peaks due to PCR-artefacts



WEBRIBO

webribo.ages.at

- Capable of differentiating ribotypes 014 and 020
 - Even **six** additional 014 subtypes can be separated
- Capable of differentiating various 001 subtypes first described by Northey et al.



WEBRIBO:

Pro/Cons

● Pros

- Database is **always up to date**
 - **follow epidemiological trends**
- **Access via internet**
- **No training** needed
- **Only** the .fsa **file is needed** to define a new ribotype
- **No additional costs**
 - no soft- or hardware is needed
- **No restriction** for sequencer, gel, size-standard or primer-set

● Cons

- .fsa data analysis algorithm **too sensitive**
- **No RAW-data** import for Beckman Systems
- PCR-Ribotype **nomenclature not in accordance** with Cardiff
 - **Subtype** nomenclature sometimes **confusing**
- **Assignment algorithm has to be improved**
- **Analysis can take some time**

WEBRIBO

webribo.ages.at

- Freely accessible internet-database for *Clostridium difficile* PCR-ribotyping
- No commercial software is needed (**Bionumerics, Genemapper, ...**)
- Real-time update
- ABI gained .fsa files can be analyzed automatically
- Reliable results regardless of any size-standard or sequencing machine used

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THANK YOU FOR YOUR QUESTIONS