ResistoXplorer: a web-based tool for visual, statistical and exploratory data analysis of resistome data

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Abstract

Background

The study of resistomes using whole metagenomic sequencing enables access to the large repertoire of resistance genes usually found in complex microbial communities, such as the human microbiome. Over recent years, sophisticated and diverse pipelines have been established to facilitate raw data processing and annotation. Despite the progress, there are no easy-to-use tools for comprehensive visual, statistical, and functional analysis of resistome data. Thus, exploration of the resulting large complex datasets remains a key bottleneck requiring robust computational resources and technical expertise, which creates a significant hurdle for advancements in the field.

Results

Here, we introduce ResistoXplorer, a user-friendly tool that integrates recent advancements in statistics and visualization, coupled with extensive functional annotations and phenotype collection, to enable high-throughput analysis of common outputs generated from resistome studies. ResistoXplorer contains three modules- the 'Antimicrobial Resistance Gene Table' module offers various options for composition profiling, functional profiling and comparative analysis of resistome data; the 'Integration' module supports integrative exploratory analysis of resistome and microbiome abundance profiles in metagenomic samples; finally, the 'Antimicrobial Resistance Gene List' module enables users to explore antimicrobial resistance genes according to function and potential microbial hosts using visual analytics to gain biological insights. Within these three modules, ResistoXplorer offers comprehensive assistance for ARG functional annotations along with their microbe and phenotype associations based on data collected from >10 reference databases. In addition, it provides support for a variety of methods

for composition profiling, visualization and exploratory data analysis, as well as extensive support

for various data normalization methods and machine learning algorithms for identification of

resistome signatures. Finally, ResistoXplorer offers also network visualization for intuitive

exploration of associations between antimicrobial resistance genes and the microbial hosts,

incorporated with functional enrichment analysis support.

Conclusions

ResistoXplorer is a web-based tool with a user-friendly interface that enables comprehensive and

real-time downstream analysis of resistome data. As such, it allows for in-depth exploration of

metagenomic datasets focusing on the intrinsic networks and correlations of antimicrobial

resistance genes and their underlying microbial determinants. ResistoXplorer will

assist researchers and clinicians in the field of AMR to facilitate discovery in large-scale and multi-

dimensional metagenomic datasets. ResistoXplorer is publicly available at

http://www.resistoxplorer.no/ResistoXplorer.

Background

Antimicrobial resistance (AMR) has emerged as a major threat to global public health and the economy [1]. Although antimicrobial resistance is a natural phenomenon, the misuse and overuse of antimicrobial agents in humans, animals and agriculture have accelerated the development and spread of antimicrobial resistance. Consequently, antimicrobials are becoming less effective in the prevention and treatment of infections. In addition, the extensive use of antibiotics has resulted in large amounts being released into the environment. This is a matter of increase concern, not only in relation to antimicrobial resistance, but also for the potential impact on the ecology of human, animal, and environmental ecosystems [2].

Microbial communities have traditionally been studied using culture techniques. Although valuable, culture-based strategies have a low-throughput, and can provide an incomplete microbial profile due to the fact that, in a majority of environments, only a limited proportion of microorganisms are cultivable. Such limitations have been largely circumvented with recent advancements in short-read based high-throughput DNA sequencing (HTS) technologies, using either amplicon targeted or whole metagenomic approaches. Amplicon approaches can be used to map both taxonomic and resistome profiles. However, one of the limitations is that only genes recognized by the specific primers can be identified. Whole metagenomics, on the other hand, enable unbiased taxonomic and antimicrobial resistance information. Such technology has been shown to provide valuable insights into the natural history of antimicrobial resistance genes (ARGs) in humans and nature [3-5], as well as how the resistome develops in early life, and how it can be affected by the use of antibiotics [6, 7]. It is also contributing to the identification of ARGs that may cross environmental and host boundaries [8], and providing unprecedented knowledge into the large reservoir of ARGs in the human, animal and environmental microbial

communities [9-14]. Currently, resistome profiles from complex and diverse microbial

metagenomes are primarily generated using whole metagenome shotgun sequencing in which the

total DNA extracted from a microbial community is sequenced. The resulting DNA fragments can

be analyzed using read or assembly-based approaches to characterize their resistome composition

[15]. These derived sequencing datasets are both large (amount) and complex (structure), causing

considerable 'big data' challenges in downstream data analysis.

The main computational effort in resistome analysis of metagenomic datasets so far has focused

on processing, classification, assembly and annotation of sequenced reads. This has led to the

development of a number of excellent bioinformatic pipelines and tools for detecting and

quantifying antimicrobial resistance genes in metagenomes [15-17]. However, there is still no clear

consensus with regards to standard analysis pipelines and workflows for high-throughput analysis

of AMR metagenomic resistome data [17, 18]. Nonetheless, the outputs from most of these

pipelines can be summarized as a data table consisting of feature (ARGs) abundance information

across samples, *i.e.* resistome profiles, along with their functional annotations and sample

metadata. For most researchers, the fundamental challenge in data analysis can often be centered

on how to understand and interpret the information in the abundance tables especially within the

context of different experimental factors and annotations.

Resistome data analysis can be separated into four main categories: (i) composition profiling- to

visualize and characterize the resistome based on approaches developed in community ecology

such as alpha diversity, rarefaction curves or ordination analysis; (ii) functional profiling- to assign

antimicrobial resistance genes into different functional categories (e.g. Drug class, Mechanism) to

gain better insights regarding their collective functional capabilities; (iii) comparative analysis- to

identify features having a significant differential abundance between studied conditions and (iv)

integrative analysis- to integrate the resistome and taxonomic data to understand the complex

interplay and potential associations between microbial ecology and AMR. The computational

methods and approaches to perform such analysis are fairly limited. The first category of analysis

can be more straightforward to perform, but the last three are challenging.

First of all, the metagenomic abundance data is often characterized by differences in library sizes,

which requires correction. To address this issue, researchers often employ two common

normalization approaches prior to analysis: subsampling the reads in each sample to the same

number (rarefying) or rescaling the total number of reads in each sample to uniform sum (using

proportions). The former may entail the loss of valuable information, while the latter could lead to

issues related to data compositionality [19]. In addition to uneven library sizes, metagenomics data

are also characterized by sparsity, over-dispersion, zero inflation and skewed distribution [20, 21].

Such unique features make standard parametric tests and most methods established in other omics

fields unsuitable for comparative analysis. To address these challenges, non-parametric

permutation-based approaches have been adopted to identify significant features in metagenomic

abundance data [22, 23]. Even though robust, these approaches are constrained by lack of statistical

power, as well as inefficiency to model confounding factors, and inability to accommodate

intricate experimental designs.

Overall, development of statistical models that account for features of metagenomic data or use of

methods to transform data to have distributions that fit standard test assumptions is recommended.

A variety of strategies have propelled empirical development in these directions. For instance,

metagenomeSeq algorithm incorporates cumulative sum scaling normalization and a zero-inflated

Gaussian (ZIG) mixture model to reduce false positives and improve statistical power for

differential abundance analysis [24, 25]. It has also been demonstrated that algorithms developed

for RNA-seq data such as edgeR and DESeq2, along with their respective normalization methods,

outperform other approaches used for metagenomic abundance data [25-28]. These standard

strategies are widely employed, but have recently been questioned due to the compositional nature

of whole metagenomic sequencing data [29, 30]. To address this issue, several Compositional Data

Analysis (CoDA) approaches to analyze sequencing datasets have been recently proposed [31, 32].

ALDEx2 and ANCOM are two CoDA methods that integrate log-ratio transformation to explicitly

deal with data compositionality while performing differential abundance testing [33, 34].

Naturally, the choice of methodology depends on the research question, and one may be interested

in comparing results obtained by different analytical methods. Both standard and CoDA statistical

approaches have been implemented as R packages. Although flexible, learning R in order to use

these methods can be challenging for most clinicians and researchers.

In addition to the challenges described above, analytical steps have another complexity layer

represented by the wide variety of reference databases to identify and characterize the

antimicrobial resistance genes from metagenomic data. These differ considerably in the quality of

data presented, as well as the scope of resistance mechanisms and the type of information provided

[15]. This can influence the accuracy of *in silico* characterization of resistome data, which depends

substantially on the comprehensiveness and quality of reference databases [15, 17, 35]. Typically,

resistome profiles are analyzed by mapping ARGs either to their respective class of drugs to which

they confer resistance (Class-level) or to their underlying molecular mechanism of resistance

(Mechanism-level). Analyzing resistomes at such high level categories enable researchers to gain

more biological, actionable, and functional insights together with a better understanding of their

data. However, these functional levels and categories, along with their classification scheme, vary

largely between the databases [18]. Additionally, depending upon the database, users need to

manually collect and curate such information and then generate separate abundance tables for each

functional level. Hence, collecting appropriate functional annotation information for hundreds of

ARGs in resistomes for functional profiling and further downstream analysis can be confusing,

arduous, time-consuming and error-prone.

Finally, some AMR reference databases may also provide information regarding the microbial

host that harbor or carry these reference ARGs. Information about such relationship can be

complex as one microbe can carry multiple ARGs and single ARGs can in turn be present across

multiple microbes. To explore such intricated 'multiple-to-multiple' relations, one option is to use

a network-based visualization method. This approach, coupled with suitable functional annotations

and enrichment analysis support, would have the potential to provide better interpretation of AMR

resistance mechanisms and inform on possible dissemination routes of antimicrobial resistance

genes. It is straightforward to identify key players from a network perspective, for instance, by

looking for those ARGs that are found in multiple microbes or by identifying those microbes that

simultaneously contain multiple ARGs of interest. Currently, there are no web-based tools that

provide such functionality.

To address the above challenges as well as to meet recent advances in resistome data analysis, we

have developed ResistoXplorer, a user-friendly, web-based, visual analytics tool to assist

clinicians, bench researchers, and interdisciplinary groups working in the AMR field to perform

exploratory data analysis on abundance profiles and resistome signatures generated from AMR

metagenomics studies. The key features of ResistoXplorer include:

• Support of a wide array of common as well as advanced methods for composition profiling,

visualization and exploratory data analysis;

• Comprehensive support for various data normalization methods coupled with standard as

well as more recent statistical and machine learning algorithms;

• Support of a variety of methods for performing vertical data integrative analysis on paired

datasets (*i.e.* taxonomic and resistome abundance profiles)

• Comprehensive support for ARG functional annotations along with their microbe and

phenotypes associations based on data collected from >10 reference databases;

• A powerful and fully featured network visualization for intuitive exploration of ARG-

microbe associations, including functional annotation enrichment analysis support.

ResistoXplorer also contains manuals to assist users to easily navigate through different analysis

tasks. Collectively, these features consist of a comprehensive tool suite for statistical, visual and

exploratory analysis of data generated from AMR metagenomics studies. ResistoXplorer is freely

available at http://www.resistoxplorer.no/ResistoXplorer.

Implementation

ResistoXplorer is implemented based on Java, R and JavaScript programing languages. The

framework is developed based on the Java Server Faces technology using the PrimeFaces

(https://www.primefaces.org/) and BootsFaces (https://www.bootsfaces.net) component library.

The network visualization uses the sigma.js (http://sigmajs.org/) JavaScript library. Additionally,

D3.js (https://d3js.org/) and CanvasXpress (https://canvasxpress.org/) JavaScript libraries are

utilized for other interactive visualization. All the R packages for performing back-end analysis

and visualization are mentioned in the 'About' section of the tool. At the start of the analysis, a

temporary account is created with an associated home folder to store the uploaded data and analysis

results. All the analysis results will be returned in real-time. Upon completing their analysis

session, users should download all their results. The system is deployed on a dedicated server with

4 physical CPU cores (Intel Core i5 3.4GHz), 8GB RAM and Ubuntu 18.04 LTS was used as the

operation system. ResistoXplorer has been tested with major modern browsers such as Google

Chrome, Mozilla Firefox, Safari and Microsoft Internet Explorer.

Results and Discussion

ResistoXplorer consists of three main analysis modules. The first is the **ARG List** module that is

designed to explore the functional and microbial host associations for a given list of antimicrobial

resistance genes (ARGs) of interest. The second is the **ARG Table** module, which contains

functions for analyzing resistome abundance profiles generated from AMR metagenomics studies.

Lastly, the **Integration** module enables users to perform integrative analysis on the paired

taxonomic and resistome abundance profiles to further explore potential associations coupled with

novel biological insights and hypotheses. Figure 1 represents the overall design and workflow of

ResistoXplorer. We recommend users to try out our example datasets to get familiar with the basic

steps and key features of the tool before proceeding with analysis of their own data. Detailed

explanation for all the steps are provided in the corresponding manuals.

Data upload and processing

Overview of data inputs

The three analysis modules (ARG List, ARG Table and Integration) are represented as three

interactive buttons in ResistoXplorer. Users must choose an analysis path based on their input. The

input can be uploaded in two different ways – by entering a list of antimicrobial resistance genes

(ARGs) or by uploading an ARG abundance table along with a sample metadata file containing

group information. In the latter case, the files can be uploaded as a tab-delimited text (.txt) or in

comma-separated values (.csv). Further, users must also provide the annotation information of

ARGs either by uploading a file (.txt or .csv) or by just selecting the appropriate database used for

their annotation. Additionally, the Integration module requires users to also upload a taxa

abundance table in the same formats. The taxonomic and functional annotation files are optional

in case of integrative analysis. Users can go to the corresponding 'Manuals' and 'Data Format'

section, or download the example datasets for more details.

ARG-functional annotations collection

The annotation information and the classification scheme for reference ARGs (or sequences) are

collected from 10 well-annotated and widely used AMR databases: ResFinder [36], CARD [37],

ARDB [38], ARG-ANNOT [39], MegaRes [18, 40], AMRFinder [41], SARG [42], DeepARG-

DB [43], ARGminer [44] and Resfams [45]. Further, this annotation information is organized into

tables containing the reference ARGs in rows and their functional annotation levels across columns

for each of the databases. The headers (names) for reference ARGs are annotated as in the chosen

database. Some of the annotation levels having several multiple functional category assignments

for ARGs were removed from the tables to avoid false counts inflation during downstream

analysis. Additional manual curation for functional category naming and data redundancies were

performed in some of the databases. Further, we also structured functional annotation information

from the BacMet [46] database and antimicrobial peptide (AMP) resistance gene dataset [47] to

enable users to perform functional profiling and downstream analysis of antibacterial

biocides/metals and AMP resistance genes abundance profiles. The tables are stored in RDS file

format for less storage space and fast retrieval of data. Users can use such table to annotate their

resistome profile directly rather than manually collecting the functional annotation information

from their corresponding database. The 'Data Format' and 'Resources' pages provide a detailed

description on the format, structure of annotation table and database statistics, together with the

links to allow users to download the example annotation structure available for the individual

database.

Data filtration and normalization

Features having very low counts or abundance across all the samples cannot be discriminated from

sequencing errors or low-level contamination, and also interfere with some statistical and

biological approximations. Hence, such features must be removed from the data before performing

downstream analysis. ResistoXplorer automatically removes features corresponding to all the

zeros or features only present in one sample. This type of minimally cleaned data is used for alpha

diversity analysis. For all other types of downstream analysis, further data filtration is required.

By default, features are filtered based on prevalence and abundances levels. Users can also choose

to remove low abundant features by setting a minimum count cutoff based on their mean or median

value. Conversely, some features remain constant in their abundances throughout the experimental

conditions or across all the samples. These features are implausible to be informative in the

comparative analysis. Users can exclude such features based on their inter-quantile ranges,

standard deviations or coefficient of variations [48]. In case of integrative analysis, users can also

choose to apply different data filtration criteria for both taxonomic and resistome abundance data.

After data filtering, users must perform data normalization to remove the systematic variability

between samples. Currently, ResistoXplorer offers three categories of data normalization-

rarefying, scaling and transformation, based on various widely used methods for metagenomic

abundance data. In addition, ResistoXplorer supports other normalization methods like centered

log-ratio (clr) and additive log-ratio (alr) transformation to facilitate compositional data analysis.

The normalized data is used for exploratory data analysis including ordination, clustering,

comparative and integrative analysis. In the integrative analysis module, taxonomic and resistome

datasets are normalized using the same approach. Applying suitable normalization methods is

critical for identifying differentially abundant features, and reduce false positive rates [25].

Composition profiling

Visual exploration

ResistoXplorer allows users to visually explore the resistome based on various intuitive

visualization approaches used for metagenomic data. For instance, users can visualize resistome

abundance data while simultaneously showing the functional hierarchical relationships and

connectivity between features using an interactive sankey diagram, zoomable sunburst or treemap

graphics (Figure 2B). In case of treemap, users can click a particular rectangular block of interest

to further inspect its compositions at a lower functional level. The abundances can be represented

as either absolute counts or relative proportions. However, such visualizations are more suitable

for resistome profiles having an acyclic and hierarchical functional annotation structure.

Abundance profiles can also be viewed at different functional levels using other common

visualizations such as stacked area or stacked bar plot (Figure 2A). The plot is organized by

experimental factors to help visualize the differences in resistome composition across different

conditions.

Diversity profiling

The resistome diversity profiling is implemented mainly based on the R vegan and phyloseq

packages [49, 50]. Currently, users can perform alpha diversity (within-sample) analysis using

eight common richness and evenness-based (or both) diversity measures. The results are

represented in the form of a dot plot for individual samples and box plots for each sample group

(Figure 2C). The corresponding statistical significance is calculated automatically using either

parametric or non-parametric tests. The analysis can be performed at different functional levels

based on the available annotations. Additionally, the reliability of estimated diversity in samples

can be assessed through rarefaction curves in which the number of unique features (ARGs)

identified is plotted against the sequence sample size (Fig. 2D).

Ordination analysis

The ordination analysis function allows users to explore and visualize the similarities or

dissimilarities between samples based on their composition at different functional levels. The

dissimilarity can be calculated using five distance measures. Currently, three widely accepted

ordination methods are supported, including principal coordinate analysis (PCoA), non-metric

multidimensional scaling (NMDS) and principal component analysis (PCA). In particular, users

can follow a CoDA ordination approach by performing PCA on the centered log-ratio transformed

data. The corresponding statistical significance is calculated using one of the three multivariate

statistical testing methods with Analysis of Group Similarities (ANOSIM) as the default option.

The results are represented as both 2D and 3D sample plots. The samples visualized in these

ordination plots are colored based on metadata or their alpha diversity measures to help users

identify any underlying patterns in the datasets (Figure 2F).

Comparative analysis

Differential abundance testing

This section enables users to perform statistical testing to identify features that are significantly

different in abundance across sample groups. ResistoXplorer supports standard tests, such as

edgeR [28], DESeq2 [27], metagenomeSeq [24], LEfSe [23], as well as more recent CoDA

univariate analysis approaches such as ALDEx2 and ANCOM [34]. EdgeR and DESeq2, are

broadly used and generally considered as robust and powerful parametric statistical approaches.

They fit a generalized linear model and assume that read counts follow a negative binomial

distribution to account for the features of count data. LEfSe uses the differential abundance

between groups. The CoDA methods perform statistical testing on features log-ratio rather than

their actual count abundances to deal with the compositional nature of sequencing data. ALDEx2,

for instance, performs parametric or nonparametric statistical tests on log-ratio values from a

modeled probability distribution of the data and returns the expected values of statistical tests along

with effect size estimates. ANCOM tests the log-ratio abundance of all pairs of features for

differences in means using nonparametric statistical tests. Both the standard and CoDA methods,

except LEfSe, use their own specific normalization procedures while conducting statistical testing.

For instance, the relative log expression (RLE) normalization is used for DESeq2, and the centered

log-ratio transformation is applied for ALDEx2. The differential analysis can also be performed at

different functional levels.

The results from the differential analysis are displayed as a table. By default, the result table will

show a maximum of 500 top features ordered according to their P-values. The significant features

(if present) are automatically highlighted in orange color. Further, users can also see a boxplot

summary for any feature of interest by just clicking the 'Details' icon. Since different statistical

approaches may generate divergent P-values, it is often recommended to compare and visualize

results using more than one statistical approach, as to increase confidence in the interpretation of

results.

Machine learning-based classification

Prediction of microbiome signatures using machine learning algorithms has been gaining more

recognition and shown to perform well in recent resistome data analyses and classifications [6, 9,

51]. ResistoXplorer provides two such powerful supervised classification methods - Random

Forest [52] and Support Vector Machine (SVM). In particular, the Random Forest algorithm uses

an ensemble of classification trees (forest) where each tree is grown based on a random subset of

features from a bootstrap sample at each branch. The final class prediction is based on the majority

vote of the ensemble. The unbiased estimate of classification (OOB) errors is obtained by

aggregating cross-validation results using bootstrapped samples while the forest is being

constructed. Random forest also measures the importance of each feature based on the increase of

the OOB error when it is randomly re-shuffled. Alternatively, the SVM algorithm seeks to find a

nonlinear decision function (hyperplane) by projecting data into higher dimensional feature space

and separating it by means of a maximum margin hyperplane [53]. The SVM analysis is performed

using recursive feature selection and sample classification via linear kernel [54]. Features are

selected based on their relative contribution in the classification using cross-validation error rates.

The features which hold less variance (least significant) are removed in the successive steps. This

procedure generates a series of SVM models (levels). The features used by the best model are

considered to be important and ranked based on their frequencies of being selected in the model.

Figure 2K shows the classification performance of SVM with regards to decreasing number of

variable (levels).

Other features

There are several other valuable functions implemented in ResistoXplorer for exploratory analysis

of resistome data. Users can perform core resistome analysis to detect core set of features present

in samples or sample groups based on their abundance and prevalence level (|Figure 2E).

ResistoXplorer also supports commonly used correlation analysis and hierarchical clustering. The

results from hierarchical clustering can be visualized using heatmaps (Figure 2G) and dendrograms

(Figure 2H). For publication purposes, all visualizations can be downloaded as either Scalable

Vector Graphics (SVG) or Portable Document Format (PDF) files.

Integrative analysis

The main goal of this module is to explore and unveil potential hidden correlations between the

microbiome and resistome using a variety of integrative data analysis approaches. Such analyses

have been increasingly used to explore the associations between the bacteria and ARGs in different

environments [13, 55, 56]. Currently, ResistoXplorer offers several advanced and commonly used

univariate and multivariate statistical methods for data integration and correlation analysis.

Global Similarity analysis

This section allows users to determine the overall similarity between the microbiome and resistome

dataset using two multivariate correlation-based approaches - Procrustes analysis (PA) and

Coinertia analysis (CIA). The analysis can be performed at various functional and taxonomic

levels based on available annotations. These functions currently support five common distance

measures. The ordinations from the distance matrices can be calculated using either PCoA, PCA

or NMDS. In case of PA, the microbiome ordination is scaled and rotated onto the resistome

ordination to minimize the sum of squared differences between the two ordinations. For the CIA,

the microbiome and resistome ordination are constrained so that the squared covariances between

them are maximized to measures the congruence between datasets. The results are represented

using both 2D and 3D ordination plots, where samples are colored and shaped based on the datasets

(Figure 3A). Users can also color the samples based on different experimental factors to identify

some patterns or gain biological insights. The corresponding correlation coefficient and P-value

from both these analyses are estimated automatically to assess the strength and significance of

association between the two datasets. The correlation coefficient ranges from 0 and 1, with 0

suggesting total similarity and 1 total dissimilarity between the two datasets.

Omics data integration approaches

ResistoXplorer offers other multivariate projection-based exploratory approaches such as

regularized canonical correlation analysis (rCCA) and sparse partial least square (sPLS) for the

integration of microbiome and resistome data. These approaches aim at highlighting the

correlations between high dimensional 'omics' datasets. They are implemented primarily based on

the R mixOmics package [57]. Users can choose or tune the number of components and

regularization parameters for rCCA. In the case of sPLS, all variables are selected in both datasets

by default. In addition to sample plots, other variable plots like clustered image maps (Figure 3C)

and correlation circle plots (Figure 3D) are displayed to facilitate the interpretation of the complex

correlation structure between datasets.

Pairwise microbe-ARG correlation analysis

This section enables users to determine if there are strong relationships (co-occurrence patterns)

between individual microbial taxa (microbiome) and ARGs (resistome) using univariate

correlation analysis. Users can perform such analysis using four different types of approaches,

including Spearman, Pearson, CCLasso and Maximal Information Coefficient. In particular,

features (ARGs or taxa) that are that not present in half of the samples are removed from both the

datasets to eliminate an artificial association bias before performing Pearson correlation analysis.

The analysis can be conducted on taxa at their different taxonomic annotations (i.e., phylum, genus

and species) and on resistome at different functional levels (e.g., class, mechanisms, ARG, etc.)

based on available annotations. Users can select strong and significant pairwise correlations using

a combination of absolute correlation coefficients and adjusted P-value. The results are represented

as a co-occurrence network (Figure 3E) with each node indicating either a microbial taxon or a

resistance determinant (ARG). The nodes can be sized based on their network topological

measures (degree and betweenness). Users can double click on a node to highlight its

corresponding correlated nodes in a network. The width and color of an edge indicate the strength

and direction of the correlation between two nodes. The nodes are colored as well as shaped

according to the dataset. The underlying correlation matrices and network centrality-based

measures are also available to download.

ARGs-microbial host network exploration

ARG-microbial host association data collection

ResistoXplorer currently supports four reference databases (ResFinder [36], CARD [37], ARDB

[38] and BacMet [46]) and a recently published AMP dataset [47] for network-based microbial

host associations exploration of ARGs. These primary databases contain direct or indirect

information regarding the microbial host for the reference ARGs. In the latter case, the information

on microbial host associated with each of the reference ARGs has been collected from their

corresponding GenBank Accession number using a combination of text mining and manual

curation, like in ResFinder and ARDB. Further, this information has been manually annotated to

improve name readability and to remove redundancy. Moreover, the available functional

annotation information of ARGs were also collected and organized into sets to facilitate

enrichment analysis.

ARG–microbial host association table and network creation

The uploaded list of ARGs are searched against the selected target database. This list can comprise

significant ARGs detected in differential abundance testing or ARGs identified through high-

throughput qPCR. The results will be represented as an association table with each row

corresponding to a particular reference ARG (sequence) and its potential microbial host. When

available, the table also provides other association information along with hyperlinks to the

corresponding GenBank Accession number and PubMed literature. Users can directly remove each

row by clicking the delete icon in the last column to keep only high-quality associations supported

by literature or experimental evidence. The resulting ARG-host associations are used to build the

default networks. Since not all the nodes will be connected, this approach may lead to the

generation of multiple networks. The statistics of nodes and edges are provided for users to have

an overview of the size and complexity of the generated networks. Further, users can also filter the

nodes based on their topological measures (degree and betweenness) in case of large networks for

better interpretation.

Network visualization and functional analysis

The resulting networks are visualized using HTML5 canvas and JavaScript-based powerful and

fully-featured visualization system. This system is implemented based on a previously published

visual analytics tool [58]. It is comprised of three main components - the central network

visualization area, the network customization and functional analysis panel on the left, and the

right panel containing a node table (Figure 4). Users can intuitively visualize and manipulate the

network in the central area using a mouse with a scroll wheel. For example, users can scroll the

wheel to zoom in and out the network, hover the mouse over any node to view its name, click a

node to display its details on the bottom-right corner or double click a node to select it. The

horizontal toolbar to the top exhibits basic functions to manipulate the network. The first is the

color picker to enable users to choose a highlight color for the next selection. Users can also select

and drag multiple nodes by using the dashed square icon in the toolbar.

The network customization panel provides various options to configure the general visualization

features of the default network or to specify the range of mouse operation. The 'Layout' option

enables users to perform automatic network layout using different algorithms; the 'Background'

option enables users to select between white and black background. The range of mouse operations

during highlighting and dragging-and-dropping can be varied using the 'Scope' option. For

instance, in 'single node' mode, only the node which has been clicked or dragged will be

highlighted or affected, whereas all the nodes that are being selected by the users will be affected

in the 'all highlights' mode. Additionally, the 'Download' option allows users to either save the

current network in different formats or to download the network file in GraphML format for

visualization in other software. The node table on the right panel displays the ARGs and their

microbial hosts along with corresponding network topological measures. The corresponding

abundance values, if available, will also be presented in the last column. Users can directly click

on any row of interest to select, and the network view will automatically zoom to the related node.

The bottom right panel provides more detailed info related to the node(s) being highlighted or

currently selected on the network.

To provide some high-level biological insights about data, ResistoXplorer also supports functional

enrichment analysis of the ARGs present within the current network using hypergeometric tests.

The categories or levels at which enrichment analysis can be performed will be based on the

initially selected database. The enrichment analysis results are shown as a table on the left panel.

By clicking on a row of the result table, users can highlight all the nodes related to enriched

function within the network. There are also several other options and functionalities present, which

allow users to intuitively explore, manipulate and customize the ARG-microbial host association

networks.

Use Case

To illustrate the functionality of the tool, we have selected two recent resistome studies with

publicly available metagenomic datasets [59, 60]. These datasets have been mounted as an

example sets in the 'ARG Table' module of our tool. In Doster et al., 2018 [60], the authors have

examined the effects of tulathromycin (antimicrobial drug) on gut microbiome and resistome using

commercial feedlot cattle, which we describe here in more detail. Two groups of cattle were used,

with one untreated group, and the other treated with metaphylaxis. Fecal samples were collected

from 15 cattle within each group at two time points- baseline (Day 1) and after 11 days (Day 11).

Shotgun sequencing was performed on the extracted metagenomic DNA, and reads were aligned

to MEGARes and custom Kraken database for resistome and microbiome characterization by the

authors. The resulting resistome abundance profile is uploaded to the ARG Table module of

ResistoXplorer for further downstream analysis and exploration. Since the reads were annotated

using the MEGARes database, we have directly selected the precompiled functional annotation

information of the corresponding database to annotate and classify ARGs (gene accessions) at

higher functional levels. All the ARGs are mapped and classified at three functional hierarchical

levels - class, mechanism and group as per the MEGARes classification scheme. We first

compared the resistome alpha diversity at the mechanism level; the Shannon diversity indices of

the treatment group decrease over time, but the diversity changes are not prominent in the untreated

group (Figure 2C). The composition profiling is carried out to explore and represent the gut

resistome of cattle. As shown in Figure 2A, the resistome composition at the class level is

dominated mainly by the ARGs that confer resistance to tetracycline and the macrolide-

lincosamide-streptogramin (MLS) class of antibiotics in all the samples. The hierarchical

composition profiling using the Sankey diagram showed that almost all of the ARGs that belongs

to tetracycline-class confer resistance through ribosomal protection proteins. In contrast, most of

the ARGs that belong to the MLS-class confer resistance through macrolide efflux pumps.

Moreover, the resistome composition at different functional levels was quite similar between

treated and untreated cattle (Figure 2B). The ordination analysis at AMR mechanism level using

PCA (CoDA-based) and NMDS indicated that the resistome composition of treated and untreated

groups were significantly different between time points (ANOSIM: R = 0.49, P-value < 0.05;

Figure 2F and 2G). We also performed differential abundance testing on fecal resistome profile

using metagenomeSeq and ALDEx2 (CoDA-based) at class, mechanism and group level. No

significant features were found to be differentially abundant between treatment groups using both

these methods at all levels. All these analyses confirm and replicate the previous findings and

results of the original publication. We also performed additional analyses of the data to highlight

the utility and exploratory capabilities of ResistoXplorer. The rarefaction curve analysis indicated

that enough sequencing depth was achieved to describe the true ARG richness in all the

metagenomic samples (Figure 2D). The heatmap showed that most of the features (except TETQ,

MEFA, TETW and TETO) at group level have a very low abundance and sparse representation

across all the 60 samples. The distinct abundance pattern of TET40 group is observed when

comparing the Day1 with Day11 for both the treatment groups (Figure 2G). The hierarchical

clustering analysis showed that samples belonging to both treated and untreated groups are

clustered effectively based on time points (Figure 2H). The ARGs belonging to tetracyclines and

MLS classes comprise the core resistome in cattle, based on their abundance and prevalence level

(Figure 2E). Additionally, both Random Forest and SVM algorithms suggested that the treatment

groups could not be predicted with high accuracies based on the resistome profiles of fecal

samples, confirming the findings that tulathromycin does not seem to influence the gut resistome

in cattle (Figure 2I and 2J).

Furthermore, the bacterial and ARG abundance profiles are uploaded to the Integration module of

ResistoXplorer to explore the relationship between the fecal microbiome and resistome in cattle.

The application of Procrustes analysis suggested that there were no significant overall similarities

between the resistome and bacterial abundance profile (M2 = 0.23, P-value > 0.05; Figure 3A).

However, the results from Co-inertia analysis indicated that resistome and bacterial composition

are moderately correlated with statistical significance (RV coefficient = 0.47, P-value < 0.05;

Figure 3B). To deal with the result discrepancies between approaches, we also investigated the

pairwise associations between individual taxa (microbiome) and ARGs (resistome) using

Spearman and Pearson correlation analysis. The results showed that no significant and strong

pairwise correlations (absolute correlation coefficient > 0.7; adjusted P-value > 0.05) were

identified between any taxa (phylum level) and ARG (group level) (Figure 3E and 3F), suggesting

that the gut resistome was not correlated and structured by bacterial composition.

Comparison with other tools

A variety of tools or pipelines have been developed in recent years to support resistome analysis

of metagenomic data [18, 36, 42, 43, 61-63]. Most of these tools have been designed primarily for

raw reads processing and annotation, with limited or no support for interactive visual exploration

and downstream analysis. ResistoXplorer complements these tools and resources by providing

comprehensive support for statistical, visual and exploratory analysis on the metagenomic

resistome data. AMR++ Shiny [18] is one option of web or locally installable application with a

graphical user interface. More recently, resistomeAnalysis [64] R package also provides the

possibility for simplified visual exploration and downstream analysis of resistome abundance

profile. Based on the detailed comparisons among those tools (Table 1), it is clear that

ResistoXplorer offers a unique set of features and functions with regards to comprehensive

statistical and exploratory data analysis, visualization, integrative data analysis and ARG-

microbial network visual analysis.

Limitations and Future directions

The ARG table module can be used for visualization and analysis of resistome profiles

characterizing different genetic determinants present within an AMR reference database.

However, ResistoXplorer does not allow users to choose multiple precompiled functional

annotation databases to analyze the entire ecologies (antimicrobial drugs, biocide, metal and other

resistance drivers) of resistance determinants. More importantly, the functional annotations

collected in ResistoXplorer mainly depends on the information and classification scheme present

in the original databases. Hence, there might still be some acyclic and hierarchical functional

annotation structure in databases, which users need to curate for accurate count-based analyses.

The biocuration of ARGs and their functional annotation structure for the supported databases is

beyond the scope of the study. Also, the ARG-microbial host associations module is intended for

visual exploration, and these associations are limited by the type and quality of information

available in the databases. Currently, ResistoXplorer offers limited functionalities and features for

vertical data integration and pairwise correlation analysis. As most of the advanced approaches for

performing this type of analysis on multidimensional datasets are based on computationally

intensive re-sampling (cross-validation) and permutation-based approaches to calculate statistical

significance, which adds layers of complexity and computational power demands that often

challenging for a real-time interactive web application. In future versions, we plan to continuously

update the expand our database and analysis support for exploration of mobilome, virulome and

other resistance determinants of relevance in AMR metagenomic studies.

Conclusion

Whole metagenomic sequencing studies are providing unparalleled knowledge on the diversity of

resistomes in the environment, animals and humans, and on the impact of interventions, such as

antibiotic use [6, 7, 9-14]. The analyses are usually exploratory in nature, and require bioinformatic

skills, thus increasing costs, and preventing full data exploration. Therefore, it is critical to assist

researchers and clinical scientists in the field to easily explore their own datasets using a variety

of approaches, in real-time and through interactive visualization, to facilitate data understanding

and hypothesis generation. ResistoXplorer meet these requirements by offering comprehensive

support for composition profiling, statistical analysis, integrative analysis and visual exploration.

ResistoXplorer will continuously be updated to follow the advancements in approaches for

resistome analysis. We believe ResistoXplorer has the potential to find large applicability as a

useful resource for researchers in the field of AMR.

Figure Legends

Figure 1: ResistoXplorer flow chart. ResistoXplorer accepts resistance gene list and ARG/taxa

abundance tables as input data. Three successive steps are performed - data processing, data

analysis, and result exploration. The accompanying web interface proffers a varied suite of options,

and generates several tables and graphics to enable users to intuitively go over the data analysis

tasks.

Figure 2: Example outputs from ARG Table module of ResistoXplorer. (A) A stacked bar chart

showing class level resistance abundance profiles across samples. (B) A Sankey diagram showing

the resistome abundance profile of treated (left) and untreated (right) cattle group at hierarchical

functional levels including class, mechanism and group. (C) A box plot summary of the Shannon

diversity index at mechanism level in different treatment groups across sampling time points. (D)

A rarefaction curve showing the number of unique ARGs identified in each sample as a function

of sequence sample size. (E) A heatmap showing the core resistome of cattle analyzed at class

level. (F) A 3D PCA plot with sample colors based on time points. (G) A 3D PCoA plot with

sample colors with regards to different treatment groups and time points. (H) A clustered heatmap

showing the variation of resistome abundance at group level in samples organized based on time

point. (I) A dendrogram showing the clustering of samples with colors based on treatment and

time point. (J) and (H) A graphical summary of the classification performance on different

treatment groups using the Random Forests and SVM algorithm, respectively.

Figure 3: Example outputs from Integration module of ResistoXplorer. (A) A 3D NMDS plot

from Procrustes analysis with samples shape and color with regards to datasets. (B) A 3D PCoA

plot from Coinertia analysis, with the length of lines connecting two points indicates the similarity

of samples between two datasets. (C) A clustered image heatmap showing the correlations between

and among taxa (phylum level) and ARGs (group level). (D) A correlation circle plot showing the

correlation structure of features (taxa/ARGs) present in two datasets. (E) and (F) A co-occurrence

network showing the strong and significant pairwise correlations between taxa (phylum level) and

ARGs (class level) identified using Spearman and Pearson correlation analysis, respectively.

Figure 4: A screenshot of ResistoXplorer network visual analytics system, The view is divided into three main compartments with the network visualization (toolbar on top) at the center, the node table on the right and the network customization panel together with functional annotation table on the left. Users can easily highlight and manually organize different groups of nodes based on either their annotations or connectivity patterns. It is straightforward to identify those ARGs that are found in multiple microbes, or those microbes that simultaneously contain multiple ARGs of interest.

Tables

Table 1: Comparison of ResistoXplorer with other web-based tools (except resistomeAnalysis R package) supporting downstream analysis of metagenomic resistome data. Tools exclusively dedicated for sequence annotations are not included. The URL of each tool is provided under the table.

Tools	ResistoXplorer	AMR++ Shiny	resistomeAnalysis	WHAM!
Platform	Web-based	Web-based + locally installable	R package	Web-based
Registration	No	No	-	No
Data Processing				
Data Input	Abundance tables	Abundance tables	Abundance tables	Abundance tables (Biobakery + EBI)
Functional annotation	User-defined + collected from >10 AMR databases	MEGARes	CARD	User-defined
Filtering	Abundance, variance	Abundance (Quantile)	-	Variance

	Scaling, transformation, rarefying	Cumulative Sum Scaling (CSS), rarefying	Total Sum Scaling (TSS), proportion	Proportion, clr
Normalization	Scaling, transformation, rarefying	Cumulative Sum Scaling (CSS), rarefying	Total Sum Scaling (TSS), proportion	Proportion, clr
Composition Profiling				
Visual profiling	Stacked bar plot, stacked area, sankey diagram, zoomable sunburst, treemap	Stacked bar plot	Stacked bar plot	Interactive Stacked bar plot
Alpha diversity	Multiple	-	Richness	-
Ordination analysis	PCoA, NMDS & PCA (2D & 3D)	PCA & NMDS (Bray-Curtis) (2D)	PCoA (2D)	-
Comparative analysis				
Differential analysis	DESeq2, metagenomeSeq, EdgeR, LEfSe, ALDEx2, ANCOM	metagenomeSeq	DESeq2	ALDEx2
Classification	Random Forests, SVM	-	-	-
Other functions	Heatmaps, dendrogram, correlation, core resistome, alpha rarefaction curves	Heatmaps, Alpha rarefaction bar plots	Heatmaps, dendrogram, correlation, core resistome	Interactive Heatmaps, correlation
Integrative analysis	Procrustes, Coinertia, rCCA, sPLS, Spearman, Pearson, CCLasso, MIC	-	Spearman	Visual comparisons
Gene (ARG) List exploration	ARG-microbial host network visual analytics & functional analysis	-	-	-

ResistoXplorer: http://www.resistoxplorer.no/ResistoXplorer

AMR ++ Shiny: https://github.com/lakinsm/amrplusplus-shiny

resistomeAnalysis (R package): https://github.com/blue-moon22/resistomeAnalysis

WHAM!: https://ruggleslab.shinyapps.io/wham_v1/

Availability and requirements

Project name: ResistoXplorer

Project home page: http://resistoxplorer.no/ResistoXplorer

Operating system: Platform independent.

Programming Language: Java, R and JavaScript.

Other requirements: None.

License: None.

Any restrictions to use by non-academics: None.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Availability of data and materials

The 'Beef Feedlot cattle' dataset has been mounted to the 'ARG Table' and 'Integration' module

data upload page as an example dataset and is downloaded from the published article, Doster et

al., (2018) Investigating effects of tulathromycin metaphylaxis on the fecal resistome and

microbiome of commercial feedlot cattle early in the feeding period. Frontiers in microbiology, 9,

1715. The 'Pig & Broiler' example dataset contains a subset of the original data that can be found

in the supplementary information of the published article Munk P et al., (2018) Abundance and

diversity of the faecal resistome in slaughter pigs and broilers in nine European countries. Nature

Microbiology, 3.8, 898-908. Lastly, the example for 'ARG List' module is downloaded from the

original article Bengtsson-Palme, J et al., (2014) Shotgun metagenomics reveals a wide array of

antibiotic resistance genes and mobile elements in a polluted lake in India. Frontiers in

microbiology, 5, 648. All these formatted example datasets can also be downloaded from the

'Resources' section of ResistoXplorer.

Authors contributions

AD conceptualized the tool, developed the software and wrote the manuscript. RJ aided in

conceptualizing the tool, interface development, and editing the manuscript. TC aided in

implementation of the tool and editing the manuscript. FCP supervised the research, and aided in

conceptualizing the tool and editing the manuscript. All authors read and approved the final

manuscript.

Abbreviations

AMR: Antimicrobial Resistance

ARG: Antimicrobial Resistance Gene

HTS: High-Throughput Sequencing

657 AMP: Antimicrobial Peptide

658 CoDA: Compositional Data Analysis

659 ZIG: Zero Inflated Gaussian

660 CLR: Centered Log-Ratio

661 ALR: Additive Log-Ratio

662 PCA: Principal Component Analysis

663 PCoA: Principal Coordinate Analysis

664 NMDS: Non-metric Multidimensional Scaling

665 RLE: Relative Log Expression

666 PA: Procrustes Analysis

667 CIA: Co-inertia Analysis

668 sPLS: Sparse Partial Least Square

669 rCCA: Regularized Canonical Correlation Analysis

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