Calculating LRs for presence of body fluids from mRNA assay data in mixtures

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Abstract

Messenger RNA (mRNA) profiling can identify body fluids present in a stain, yielding information on what activities could have taken place at a crime scene. To account for uncertainty in such identifications, recent work has focused on devising statistical models to allow for probabilistic statements on the presence of body fluids. A major hurdle for practical adoption is that evidentiary stains are likely to contain more than one body fluid and current models are ill-suited to analyse such mixtures. Here, we construct a likelihood ratio (LR) system that can handle mixtures, considering the hypotheses H1: the sample contains at least one of the body fluids of interest (and possibly other body fluids); H2: the sample contains none of the body fluids of interest (but possibly other body fluids). Thus, the LR-system outputs an LR-value for any combination of mRNA profile and set of body fluids of interest that are given as input.

The calculation is based on an augmented dataset obtained by in silico mixing of real single body fluid mRNA profiles. These digital mixtures are used to construct a probabilistic classification method (a ‘multi-label classifier’). The probabilities produced are subsequently used to calculate an LR, via calibration. We test a range of different classification methods from the field of machine learning, ways to preprocess the data and multi-label strategies for their performance on in silico mixed test data. Furthermore, we study their robustness to different assumptions on background levels of the body fluids. We find logistic regression works as well as more flexible classifiers, but shows higher robustness and better explainability. We test the system’s performance on lab-generated mixture samples, and discuss practical usage in case work.

Keywords: Body fluid typing, mRNA profile, LR system, Machine learning, Calibration

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1 Introduction

In forensic case work, DNA profiling is an important and frequently used tool, as it has the ability to reveal the identity of a donor of a trace with high evidentiary value. It is increasingly questioned how evidentiary traces got deposited (rather than by whom), resulting in activity level evaluations. Body fluid(s) contributing to an evidentiary trace can provide such activity level information. Conventional, presumptive methods for body fluid inference include chemical, enzymatic and histological assays that
tend to be of limited sensitivity and specificity (both towards body fluids and human origin) and are often
presumptive in nature and only suitable for identification of one body fluid at a time [1,2]. Alternative
methods for body fluid inference include messenger RNA-based approaches [3–11]. These can be targeted
at organ tissues or body fluids, and both assays are applied in forensic casework [3,12,13]. Inference of
organ tissues is less frequent and mainly for objects involved in violent crimes. The body fluid assay is
most frequently used, mainly in sexual assault cases in which the presence of vaginal mucosa cells and/or
menstrual secretion is disputed. Therefore the most relevant body fluid to reliably detect are vaginal
mucosa and menstrual secretion. A complicating factor is that many if not all samples will contain a
mixture of human cell types, e.g. due to the omnipresence of skin cells.

By nature, mRNA markers have neither perfect specificity nor sensitivity [2]. As a practical solution,
a decision rule or threshold scoring system is used to make a categorical statement on the presence of
body fluids based on the number of times specific markers were observed [3,7]. These methods represent
“fall-off-the-cliff” procedures and to increase reliability, replicate RNA analysis (generally four) can be
applied [3]. In this approach, RNA data is evaluated by applying an “x = n/2” scoring system per body
fluid. Here, “x” reflects the number of observed and “n” the number of theoretically possible signals in
all replicates. Body fluids for which x ≥ n/2 are reported as “Indication for the presence of...” that
body fluid. Body fluids are reported as “No indication for the presence of...” when x = 0, and as “No
reliable statement possible” when 0 < x < n/2. This rule has a number of drawbacks, such as fall-off-
the-cliff behaviour (one more marker detected may completely change the result), an inability to chain
evaluations and an inability to be easily updated when more data becomes available [14]. Therefore,
there has been increasing interest in statistical methods [15–19], which give a probabilistic statement on
the presence of a body fluid in a sample given measurements. Unfortunately, all methods proposed make
the simplifying assumption that only one body fluid is present per sample. This makes the methods
ill-suited to evaluate mixture data, which presents a major obstacle to using these methods in practice
as most evidentiary samples may comprise more than one cell type. For example, experiments in [17]
show that the method will strongly indicate only one body fluid to be present for mixture samples, even
though the data clearly show multiple body fluids are present.

Machine learning methods have been gaining traction in a variety of (forensic) fields, due to their
flexibility and ease of use, and have been proposed for forensic mRNA analysis [18]. One drawback is that
these models and their predictions are hard to interpret, as the models often feature many parameters
that do not directly correspond to properties of the modeled system. Furthermore, these classifiers often
are not properly calibrated. An LR system is well calibrated when the LR-values it produces are not
“too large” or “too small”, or more formally, that the LR of the LRs is the LR. For LR systems based
on machine learning, a post-hoc calibration step is needed. This procedure may be relatively unknown
in forensic biology, possibly because well-fitting statistical models have long been available for forensic
DNA analysis, but they are routinely used in fields as diverse as forensic voice [20], glass [21] and cell
phone pattern analysis [22]. Although machine learning has proved useful in a variety of fields and is
now applied to diverse problems, it is unclear what its utility is in an application like forensic mRNA
analysis that has relatively small datasets.

Another problem facing probabilistic methods in forensic mRNA analysis is that it is difficult to
randomly sample from the (case-dependent) H2 population. This random sample is needed to establish
the background levels of body fluids for which H1 or H2 make no specific statements, and thus the
markers we would expect to find if the alleged activity did not take place. For example, we do not know
the background level of saliva in underpants, nor whether this depends on age, sex or socio-economic
background of the wearer. Fully defining the relevant case-dependent population is difficult and sampling
from it is often infeasible as the population should be representative with respect to traits that correlate
with the body fluids and markers we would expect to find. Such traits may include age, socio-cultural
background, physiological condition, stress and sampling site. As statistical models require an explicit
assumption on background levels to be made, previously proposed methods have made the simplifying
assumption of equal background levels. Depending on the case at hand however, the forensic scientist
may feel confident to make stronger assumptions. For example, we may reasonably assume a penile
sample to include penile skin. Any (probabilistic) method to be used in practice should not be too
sensitive to simplifying assumptions like a uniform distribution, and should support being updated with
relevant knowledge on background levels by the forensic expert.

In this paper, we construct and evaluate LR-systems based on multi-label models, i.e. models that
explicitly allow for mixtures of body fluids. We use in silico mixing, generating mRNA mixture profiles
with a computer from real single body fluid mRNA profiles. The models include a range of simple to
complex machine learning models, all calibrated to obtain LRs. We perform a sensitivity analysis to
establish the impact of differing assumptions on background levels, and test performance on mRNA profiles of mixture samples generated in the lab. We show how background levels relevant for the case can be included, and discuss practical usage and interpretation.

2 Methods & Materials

2.1 RNA profiling data

The body fluid samples used for this study were collected with informed consent of the voluntary donors whose cell material was used and the study was executed according to approved procedures. Body fluid samples were collected as described by Lindenbergh et al. [3]. DNA/RNA co-extraction, DNase treatment, reverse transcription, PCR amplification, PCR purification and detection for all RNA analyses were performed according to standardized protocols [3]. Profile analysis was performed using Genemapper ID-X version 1.1.1 (Life Technologies) with a peak detection threshold of 150 relative fluorescence units (rfu).

We obtained samples for \( k = 9 \) body fluids, and measured expression of \( p = 15 \) body fluid-specific markers (Table 1) as well as two control housekeeping markers [3,4,12]. These were either processed as single samples, or combined to yield a mixture sample. Following standard procedures [4], each sample was analyzed multiple times, yielding two to four replicate measurements per sample. Samples were discarded if fewer than half the housekeeping markers amplified. This left 212 single body fluid and 188 mixture samples of seven body fluid combinations. Note that the single samples were designed to reflect case conditions, e.g. by including degraded or low quantity samples, whereas the mixtures were constructed from good quality samples. This explains the higher detection rates for markers in the mixture set, i.e. the higher fractions seen in Table 1 (bottom). We treated the body fluids here as distinct, e.g. ‘skin’ should be read as ‘non-penile skin’, and we ignored that menstrual secretion may itself contain blood or vaginal cells. See discussion for possible improvements on this simplification.

All data and scripts used are available on github.
Table 1: Detection rates of markers, i.e. the proportion of replicates (generally four times the number of samples) in which each marker was detected, per single body fluid (top) and mixture (bottom). Note: CYP2 stands for CYP2B7P1

| Body fluid (@samples) | HBB | ALS2 | CD93 | HTN3 | STAT9 | BPIFA1 | MUC4 | MYOZ1 | CYP2 | MMP10 | MMP7 | MMP11 | SEMG1 | KLK3 | PRM1 |
|-----------------------|-----|------|------|------|-------|--------|------|-------|------|-------|------|-------|-------|------|------|
| Blood (31)            | 1.000 | 0.960 | 0.579 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.032 | 0.000 | 0.000 | 0.000 |
| Nasal mucosa (31)     | 0.008 | 0.000 | 0.440 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.008 | 0.024 | 0.000 | 0.000 | 0.000 |
| Semen fertile (28)    | 0.011 | 0.011 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.832 | 0.769 | 0.958 | 0.000 |
| Skin (18)             | 0.264 | 0.031 | 0.111 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| Vaginal mucosa (31)   | 0.009 | 0.000 | 0.157 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.014 | 0.000 | 0.000 | 0.000 | 0.000 |

Saliva+Semen fertile (32) 0.438 0.000 0.000 1.000 1.000 0.000 0.531 0.000 0.000 0.000 0.000 0.000 0.719 0.844 1.000
Saliva+Semen fertile+Vaginal mucosa (32) 0.000 0.000 0.000 0.000 0.000 0.000 1.000 1.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000
Blood+Nasal mucosa (30) 1.000 1.000 0.967 0.000 1.000 1.000 0.000 0.000 0.000 0.000 0.000 0.102 0.000 0.000 0.000 0.000
Blood+Vaginal mucosa (14) 1.000 1.000 0.714 0.000 0.000 0.000 0.000 0.000 1.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000
Saliva+Vaginal mucosa (32) 0.000 0.000 0.000 0.000 0.000 0.000 1.000 1.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000
Nasal mucosa+Saliva (32) 0.125 0.000 0.406 0.969 1.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000 0.000

Table 2: Measurement values for four replicates in three example cases.
3 Data splitting and augmentation

We randomly split the single body fluid samples into 40% ‘training data’, 40% ‘calibration data’ and 20% ‘test data’ before further processing, stratified by body fluid. The training data are used to fit a model, i.e., calculate the model parameters. After the training step, we have a model that for observations on a new sample produces a number (a ‘score’). The idea is that samples for which H1 is true will have higher scores than samples for which H2 is true. Subsequently, the calibration data are used to calibrate the score, i.e., transform the score to an LR, which is interpreted as the strength of the evidence. Thus, we have built an ‘LR system’ that for a new sample calculates the LR. Finally, the test data are used to test how well this LR system works.

For each of the training, calibration and test datasets we applied data augmentation to generate 10, 10 and 5 samples respectively for each of the $2^k$ possible combinations of body fluids. By data augmentation we mean the process of creating synthetic data representing mixtures of body fluids in silico, using mRNA marker data from real samples of single body fluids, see below and Figure 1 for details. $k=8$ for most analyses, as we exclude penile skin (see methods section), yielding a total of $2^8 \times (10 + 10 + 5) = 6400$ augmented samples.

Our data augmentation scheme is as follows. For each of the body fluids simulated to be in the mixture, we selected a single sample of that body fluid at random (with replacement) from the dataset of single samples. We then shuffled the replicates for the selected samples independently, and constructed augmented replicates by combining the first replicate for each selected sample, the second, etc. An augmented sample was constructed from a combination of augmented replicates by taking as peak height per marker the maximum peak height observed in the combined replicates for that marker (Figure 1). This reflects our assumption that mRNA signals for a marker specific for a body fluid will be little affected by the presence of another non-target body fluid, and that responses of a marker with non-target body fluids is mostly experimental noise and will not increase when multiple body fluids are present for which the marker is not-responsive. Experiments with taking the sum of the peak heights rather than the maximum (not shown here) give similar results. Lastly, for each marker, we took the average peak height over the augmented replicates to be the peak height for that marker for the augmented sample. We explored the performance of our LR system both on dichotomized and non-dichotomized data. We obtained the dichotomized data by setting the value for a marker to 1 if the peak height $\geq 150$ rfu, 0 otherwise.
Figure 1. Schematic overview of the augmentation process to generate a mixture sample (in this case blood and saliva) from single body fluid mRNA profiles for which three replicates were present.

Note that the augmented samples are not all independent, which increases the risk of overfitting of statistical models using the training data. This is ameliorated by the calibration step, which is performed on the calibration data independent from the training data. Finally, we test on augmented test data independent from the training and calibration data, and on the lab-generated mixture samples that were not used in the model construction process. The latter is also a good test of our assumptions in the augmentation process. We repeat this whole process, including the random splitting, 10 times and evaluate the metrics resulting from these 10 runs.
4 Methods

For mRNA data, formulation of the two competing hypotheses differs from classical ‘the source of the DNA is person X’/ ‘the source of the DNA is a random person (unrelated to X) from the population’ for two reasons. Firstly, rather than having a ‘reference’ individual and a background population from which random sampling is possible, we have \( k \) distinct ‘individuals’ (i.e. body fluids) which we assume to comprehensively encompass the possible sources, and little knowledge on background levels. Secondly, the sample of interest most likely results from a combination of these sources. A subset of the \( k \) body fluids will be forensically relevant. The pair of hypotheses we considered are thus:

\[
H_1: \text{the sample contains at least one body fluid of interest (and possibly other body fluids)},
\]

\[
H_2: \text{the sample contains none of the body fluids of interest (but possibly other body fluids)}.
\]

Note that the presented framework could be extended to allow a more specific defense specification, such as \( H_2: \text{the sample contains body fluid X, none of the body fluids of interest, and possibly other body fluids} \). This could be achieved by only generating samples in the data augmentation step that are consistent with either \( H_1, H_2 \) or both. See supplementary text for more details.

As background levels for the various body fluids are generally unknown, we assume a simple uniform distribution. Note that this is not needed for body fluids for which prosecution and defense agree on presence/absence, or for which prosecution and defense make no statement but the forensic scientists feels confident to make a presence/absence assumption. Blood may be an example of the first, as in some cases it is clearly visible, penile skin an example of the latter, as it can be assumed to be present or absent depending on whether we are considering a penile sample. Thus, we set the background level to 0 for penile skin, and all other body fluids to be present in 0.5 of all samples, independently. Practically, we implemented these background levels by generating mixture samples in the appropriate proportions using the data augmentation scheme described above. We tested the impact of the assumption of uniformity using a sensitivity analysis (see below).

To allow for the simultaneous presence of different body fluids, as seen in mixtures, we constructed a multi-label classifier. Like the more common multi-class classifier, a multi-label classifier predicts the presence of each of a number of classes. The difference is that the former requires the probabilities to sum to one, i.e. makes the hard assumption that exactly one class is present, whereas in the latter there is no such restriction, allowing modelling of the simultaneous presence of zero to all classes. Because of the higher number of possible outcomes, multi-label classification is often considered a harder problem than the ‘standard’ multi-class classification. For multi-label classification an adaption of ‘standard’ classifiers is usually used [23]. We explored two such adaptation strategies: the label power-set method and one-vs-rest adaptation strategy (see Fig 2). In the former, each of the \( 2^k \) mixture classes is treated as a new class. Although accurately calculating the probabilities for each of these classes is clearly very challenging, the estimates have to be less precise as we subsequently take the marginal distribution of the set of body fluids of interest. In the example of figure 2C, this means we would sum the probabilities of all urns containing blue, arriving at 0.96. In the latter, the multi-label problem is transformed into a binary classification problem for the set of body fluids of interest, taking the marginal distribution during training rather than after. In the example of figure 2B, blue would represent the set of body fluids of interests, arriving at 0.9. This strategy has the general drawback that interaction effects cannot be modelled, which may make this adaptation strategy less desirable if actual mixture mRNA profiles are available.

![Figure 2. Illustration of different classifiers (black boxes) having to classify a fictitious sample containing red and blue (discs), where possible labels are blue, red and yellow (colored urns). (A) A multi-class classification approach. (B) A one-vs-rest approach. (C) A label power-set approach.](image-url)

7
classifier cannot correctly classify a sample containing both red and blue, high certainty on red may artificially lower its prediction of blue. (B) A multi-label classifier using the one-vs-rest adaptation strategy consists of three separate binary classifiers, each predicting the presence of one color, ignoring the other two. (C) A multi-label classifier using the label power-set adaptation strategy extends the classes to all possible combinations of original labels, and uses a multi-class classifier on this expanded space.

5 Models

We explore five classification models (‘classifiers’) common in the machine learning literature: (multinomial) logistic regression (MLR) [15], multi-layer perceptron (MLP) [24], support vector machine (SVM) [25], extreme gradient boosting (XGB) [26,27] and random forest (RF) [28]. These models are explained below. All of these models provide, after fitting on training data, a score for new samples. This score is a single number. For many machine learning models, e.g. SVM, this score has no probabilistic interpretation but is simply a (transformation of a) distance measure. For models like MLR the probabilistic interpretation exists, but can only be taken at face value if we are willing to make the assumptions imposed by the model, such as independence between marker values. We thus implement the extra step of calibration, in which the score distributions on independent data under $H_1$ and $H_2$ are used to map scores to LRs (see section Calibration below). We use the Python implementation in the xgboost package [27] for XGB, and the sklearn package [25,29] for the others.

Logistic regression is a well-known model in both the statistical and machine learning literature. In the simple case of having to calculate the posterior probability $P$ on the presence of a single body fluid, the logistic regression model computes the log posterior odds as the weighted sum of the measured rfu:

$$\log_{10}\{P/(1-P)\} = \beta_0 + \sum_{i=1}^{p}\beta_i r_i$$

where $p$ is the number of markers, the observations $r$ are the (possibly dichotomized) rfu values, and the coefficients $\beta$ are parameters to be estimated. If no markers were detected, the resulting value would be equal to $\beta_0$. The coefficients can be positive or negative, indicating that detecting the associated marker increases or decreases our belief that the body fluid of interest is present. Note that for this model, the posterior probability $P$ that the body fluid is present is given by the logistic function (where we use base 10 for consistency):

$$P = 1/(1 + 10^{\beta_0 + \sum_{i=1}^{p}\beta_i r_i})$$

These posterior probabilities are based on the assumption that the prior probabilities are given by the composition in the training data: if the body fluid is present in 50% of the training data, then the prior probability is implicitly taken as 50%. In this case, the prior odds are 1 so that the posterior odds equal the LR.

When there are multiple, mutually exclusive body fluids that could be present, the model can be extended to multinomial logistic regression (MLR). Unfortunately, the equations for the LRs are no longer as neat, with the posterior probabilities $P_m$ for the $k$ body fluids given as

$$P_i = 1/(1 + \sum_{j=2}^{k}10^{\beta_{0,j} + \sum_{i=1}^{p}\beta_{i,j} r_i})$$

$$P_m = 10^{\beta_{k,m} + \sum_{i=1}^{p}\beta_{i,m} r_i} / (1 + \sum_{j=2}^{k}10^{\beta_{0,j} + \sum_{i=1}^{p}\beta_{i,j} r_i})$$

for $1 < m < k$, where $\beta_{i,j}$ is the parameter for the $i$-th marker and $j$-th body fluid. $0 < i \leq p$, $1 < j \leq k$, resulting in $p(k-1)$ parameters. This is the model suggested in de Zoete et al. [15]. We extend on this by allowing multiple body fluids to be present simultaneously, using the one-vs-rest and label power-set strategies introduced above. In the one-vs-rest strategy, we construct a binary model for the set of body fluids of interest, i.e. a logistic regression model. This has the advantage of high interpretability: for prior odds of 1, the log LR is a linear combination of the estimated parameters (equation 1). We illustrate this interpretability by showing that these coefficients behave as expected at the end of the results section (Fig 8). In the label power-set strategy, we use the same multinomial model defined above, but consider as classes the $2^k$ possible combinations of body fluids. To obtain the model output for a set of body fluids of interest, we sum the probabilities for classes that contain one or more of the body fluids of interest ($H_1$ is true), and divide by the sum of the probabilities for classes that contain none of the body fluids.
fluids of interest (H2 is true). This ratio is the score that is produced by the MLR model based on the measured rfu of a new sample.

A multi-layer perceptron (MLP) is an example of an artificial neural network, a class of statistical models loosely based on the working of the human brain. Neural networks have become very popular in machine learning due to good performance on long-standing difficult problems such as image and text analysis. A neural network typically consists of several layers of ‘nodes’, analogous to neurons in the brain. The MLP we use has two such layers, and the nodes themselves are logistic regression models. The first layer consists of 100 such models, each taking the features of the sample (i.e., the measured rfu) as input. The second layer takes the output of the nodes in the first layer as input. The output of the last layer is normalised to form the output of the MLP. The number of nodes in the output layer is equal to the number of classes, $2^k$ for the label power-set adaptation strategy or $k$ for the one-vs-rest adaptation strategy. Note that the second layer in itself forms a multinomial logistic regression model, working on the output of the first layer, a ‘non-normalised’ multinomial regression model. Thus, the MLP is an extension of logistic regression. For the MLP, the one-vs-rest strategy does not result in independent models as the first layer is shared for all $k$ classes. This means characteristics of the data do not have to be relearned for each class. Thus, a model is built that calculates a score based on the measured rfu of a new sample.

In machine learning, the vector representing the observations is called the ‘feature vector’. Here, the feature vector is the $p$-dimensional vector representing the measured rfu values of the $p$ markers. We can imagine these vectors as points in a $p$-dimensional space, the ‘feature space’. In graphical representations, the composition of the various body fluids in each sample (the ‘class’ of each sample) can be represented by the colours of the points. A support vector machine (SVM) defines a hyperplane in the (possibly transformed) feature space, aiming to separate samples of different classes and maximize the minimum distance (=margin) from any sample to the plane. The hyperplane can thus be defined in terms of the feature vectors of the samples that lie closest to it; the support vectors (i.e. the samples that are closest to the other class). We use sklearn’s implementation defaults. This means the one-vs-one adaptation strategy is used to convert this binary classifier into a multi-class classifier. This strategy is similar to the one-vs-rest strategy explained above, but rather than building $k$ binary classifiers to distinguish between a class and all other classes, $k(k-1)/2$ binary classifiers are built to distinguish between every pair of classes. Furthermore, a radial basis function is used as a kernel, and squared L2 regularization is employed to penalize non-zero parameters. Lastly, the method proposed by Platt itself a form of logistic regression calibration, is used to convert the distance to the decision boundary to a score. Thus, a score is obtained from the measured rfu of a new sample.

Both random forests (RF) and extreme gradient boosting (XGB) are examples of ‘ensemble methods’, where several simple models (decision trees) are combined to produce a well-performing model. In a decision tree, splits are made to the dataset iteratively based on a single feature, maximizing homogeneity of the split datasets. For XGB, 100 trees with a maximum number of three leaves are sequentially fitted to the residuals of the previously fit trees. For the RF, 100 trees are fitted in parallel to a bootstrapped version of the dataset, and their average predictions are taken as the final prediction.

6 Calibration

An important aspect to consider for any LR system is whether it is well-calibrated. Intuitively, a probabilistic system is well-calibrated when events for which it gives a probability of 0.9 happen 90% of the time. For example, if a weather forecasting system’s predictions of 0.9 probability of rain are followed by rain only 50% of the time, we would call the system, and the probabilities it outputs, uncalibrated. We could ‘calibrate’ the system, by adjusting the uncalibrated probability of 0.9 to the calibrated probability of 0.5. Equivalently, in a forensic context, for well-calibrated systems the property ‘the LR is the LR of the LR’ holds. Intuitively, this means that the size of the LR is coupled with the frequency of occurrence under each hypothesis. For example, if we observe a set of hundred samples taken under H2 conditions, we may expect to observe a ‘misleading’ LR of ten about ten times, and a misleading LR of hundred just once. If we observe a misleading LR of one million three times, we have a clear indication that our LR-system is calculating LRs that are off, i.e. are not consistent with expected frequencies. We then say that the LR-system is ill-calibrated. Ill-calibrated LR-systems are of use if they have discriminative power, i.e. if LRs calculated for H1 tend to differ from LRs calculated for H2. The output of any statistical model could be ill-calibrated, for example if the model assumptions deviate too much from reality.
The machine learning models introduced above have been found in the machine learning literature to provide good performance to a wide variety of problems. However, the machine learning literature usually does not focus on calibration of the output, as performance in many applications only pertains to the relative sizes of the probabilities, i.e. discriminative power. We should thus not assume these models to be well-calibrated. To emphasize that the uncalibrated LRs output by the models should not be interpreted as calibrated LRs, we refer to them as scores [33]. In order to interpret them as LRs, we need to transform them by ‘calibration’. We implemented a calibration step in which a logistic regression model is fitted on the log scores [20,34,35], see figure 3 for an illustration. The calibration step is performed for each set of body fluids of interest separately. Thus, each ‘score’ is transformed into a number that we now call LR. Fig 3(C) shows the transformation function from score to LR for an example.

Note that, for uniformity, we also apply this logistic regression step to the logistic regression model. Luckily, for the one-vs-rest adaptation strategy, the additional logistic regression calibration step preserves interpretability, as it only scales and translates the coefficients:

$$\log_{10} LR = a_0 + a_1 (b_0 + \Sigma_{i=1}^n b_i r_i) = (a_0 + a_1 b_0) + \Sigma_{i=1}^n a_1 b_i r_i = \beta_0 + \Sigma_{i=1}^n \beta_i r_i$$  \hspace{1cm} (2)

Fig 3. Illustration of calibration using logistic regression.

(A) Distribution of $\log_{10}$ scores $s$ for (orange) $H_1$ and (blue) $H_2$ samples, for a hypothetical model. If the model were well-calibrated, the scores would be interpretable as LRs. The model is discriminative as the scores discriminate between the hypotheses, but ill-calibrated as ‘the LR is the LR of the LR’ does not hold for $s$. For example, there are 10 times more $H_2$ samples that result in $\log_{10}(s) = 0$ than there are $H_1$ samples that do so, meaning $P(s = 1|H_1)/P(s = 1|H_2) = LR(s = 1) = 0.1$. Likewise $LR(s=0.1)=0.01$ and $LR(s=10)=1$. The model would be well-calibrated if all scores were 10 times smaller.

(B) The fit of a logistic regression model that outputs $P(H_1|\log(s))=P_{log\_reg}$ from the distribution in (A). Dots represent the actual data points (some random noise in the x-axis was added just to be distinguishable).

(C) The mapping from $\log(s)$ to $\log(P_{log\_reg}/(1-P_{log\_reg}))$ illustrates that logistic regression gives a linear mapping in the log odds space. We refer to $P_{log\_reg}/(1-P_{log\_reg})$ as the calibrated LR.

(D) Distribution for $H_1$ and $H_2$ samples obtained after logistic regression calibration. Note that the calibration still is not perfect, as values are shrunk slightly towards $\log(LR)=1$.

7 Measuring performance

When evaluating an LR system and comparing it to others, several aspects are of interest [36]. The most obvious is discrimination, the ability of a system to tell apart the competing hypotheses, as measured by metrics such as accuracy or area under the curve of the receiver operator characteristic. These measures however ignore calibration, i.e. whether not just the ordering but also the magnitude of the LR values is what it should be. We therefore use the log likelihood ratio cost ($C_{llr}$), the empirical cross-entropy for
equal priors, a metric suggested by information theory that measures both discrimination and calibration \[37\]. Its value will be 0 for a perfect system, and 1 for a system that does not improve decision making (e.g. a system that always outputs LR=1). This means any system with a value of $C_{llr}<1$ will improve decision making, with smaller values being better.

8 Sensitivity analysis

As noted before \[15\], since we are not sampling at random from the population but assuming equal background levels for the body fluids, a deviation from this assumption may affect the performance of our LR systems. Although we cannot establish the background levels, we can explore the magnitude of this effect by constructing the same models using differing assumptions on these background levels, and assessing the differences in LR values calculated. We generated augmented datasets of the same size in which a particular body fluid is present in 0.9 rather than 0.5 of generated samples. We train a system on this dataset using the same procedure, and plot the LRs it generates against the LRs generated by the original system. A large deviation would indicate a high sensitivity which would make the method less useful in practice.

In total, we performed 10 runs of different dataset splits, each run consisting of 2 dichotomization of data x 2 multi-label strategies x 4 models = 16 settings. We show $C_{llr}$s for the set of body fluids of interest being vaginal mucosa and/or menstrual secretion. We performed the 10x 16 x 2 set of computations 3 more times, increasing the background levels of blood, nasal mucosa and skin, and compared the LRs generated by the original model with the LRs generated by the models trained on these non-uniform data.

9 Practical use and interpretation

To illustrate the use and interpretation of the best performing model, we present three more results. First, we illustrate the best performing model on illustrative cases, discuss the characteristics of the model, and how these change when the background level of penile skin is set to 1. Second, we contrast the multi-label model to previously proposed multi-class models. Third, we assessed performance of the model on 86 traces from RNA casework conducted at our laboratory between 2018 and 2020 for which the presence of vaginal mucosa and/or menstrual secretion was questioned. These have been analyzed in the context of the case using the n/2 interpretation method \[3\] summarized in the introduction. We compare conclusions given by the n/2 method and the model described in this paper.

10 Results

Results shown have vaginal mucosa and/or menstrual secretion as body fluids of interest, and logs are to base 10, unless specifically stated otherwise.

Performance for all models and multi-label strategies on dichotomized/non-dichotomized data are shown in figure 4. We see that under all scenarios tested the methods perform well, with $C_{llr}$s ranging from 0.1 to 0.7. This means that, under the assumptions of the augmentation scheme, using any of these methods will improve decision making. Generally, the models perform very similarly, with distributions of $C_{llr}$s over the 10 runs mostly overlapping. For illustration, figure 5 shows the underlying LR distributions and ROC curves (for the MLR, one-vs-rest, dichotomized method), for a number of (combinations of) body fluids of interest.
Fig 4. Distributions of $C_{llr}$ on the augmented test data for the different tested scenarios: Data (A) dichotomized or (B) not; using the ‘one-vs-rest’ strategy (5 boxplots on right) or ‘label power-set’ method (5 boxplots on left). Box colour marks the five different classifiers.
Fig 5. Performance of the MLR, one-vs-rest, dichotomized method, for six different sets of body fluids of interest, for (A, C) augmented test data and (B, D) lab-generated test data. A and B show inverse cumulative density functions of $10\log \text{LRs}$ (‘Tippett plots’) for (orange) H1 and (blue) H2. C and D show receiver operating curves, reporting on the area under the curve (AUC) and false positive (FP) and false negative (FN) rates. Note that no skin markers are present in the data, correctly leading to LR $\approx 1$ (A; bottom right) and a curve close to the diagonal (C; bottom right) when skin is assessed. As the lab-generated mixture samples contain no examples with skin, B and D bottom right have no H1 (orange) distribution, AUC or FN rate.

Figure 6 shows $C_{\text{llr}}$s when the LR systems are tested (but not trained/calibrated) on independent lab-generated mixture data. We note that for the dichotomized data (left panel) all models and both multi-label strategies perform better than on the augmented test data (i.e. show lower $C_{\text{llr}}$). This performance increase may be explained by the mixture samples being less degraded by design (see Data section). The similar performance of the two multi-label strategies on the test data (Fig 4) motivates us to focus henceforth on the one-vs-rest strategy, as it is the simpler method. In particular for the MLR model it leads to higher interpretability (see Interpretability section below).

In contrast, without dichotomization, most models perform worse, even showing $C_{\text{llr}}$ values above the critical threshold of 1 (Fig 6, right panel). This poor performance may well be due to faulty assumptions in the way we generate peak heights in the data augmentation scheme, or because the peak height distribution is different for the mixture data that were not designed to reflect case conditions.
Dichotomizing data is less sensitive to actual peak heights, which would explain the lack of a performance drop. Only the decision tree-based models (RF and XGB) show reasonable performance, albeit worse than on the dichotomized data. This is probably because such models use thresholds to handle non-categorical data, making them inherently robust to changes in peak heights. In the following we therefore focus on the dichotomization strategy, for its higher robustness.

**Fig 6. Distributions of $C_{llr}$ on the laboratory mixture data for the different tested scenarios for: Data (A) dichotomized or (B) not; using the ‘one-vs-rest’ strategy (5 boxplots on right) or ‘label power-set’ method (5 boxplots on left). Box colour marks the five different classifiers. Note that in (B), all $C_{llr}$s for the SVM were $> 1$.**
Fig 7. $\log_{10}$ LRs obtained for samples containing (orange) vaginal mucosa and/or menstrual secretion or (blue) neither of these. y-axis: training with the assumption of a background level of 90% probability for the presence of blood, nasal mucosa or skin. x-axis: training with the assumption of uniform background levels. Rows show scatterplots for the multi-layer perceptron (MLP), multinomial logistic regression (MLR), random forest (RF), support vector machine (SVM) and extreme gradient boosting (XGB) models, using dichotomization and the one-vs-rest multi-label strategy.
Figure 7 shows the LRs obtained on an augmented test set constructed with equal background levels, for models trained on augmented train data with equal background levels (uniform) or a background level of 90% probability for the presence of blood, nasal mucosa or skin respectively (with dichotomization, one-vs-rest strategy). We see that the difference between LRs obtained ranges from low (MLR and MLP) to very high (SVM). Small differences in LRs are indicative of the LR system being relatively insensitive to a violation of the assumption of equal background levels. We will focus on MLR as its performance is comparable to other models (Fig 4), its robustness is similar or better (Fig 7) and its interpretability is much higher (see below).

11 Practical use and interpretability of the MLR one-vs-rest LR system

In this section, we illustrate usage of our preferred model, logistic regression (MLR) on dichotomized data and a one-vs-rest adaptation strategy. As logistic regression is linear regression in the log odds space, the log LR can be calculated as a weighted sum of the measured markers (see equation 2). To illustrate this, consider three cases in which the presence of vaginal mucosa and/or menstrual secretion is disputed, and for which measurements for four replicates are given in table 2. We can compute the relevant log LRs from equation 2 by plugging in the coefficients given in figure 8.

For case 1 only the blood markers are observed: 3/4 for HBB and 4/4 for both ALAS2 and CD93 (Table 2). Although we expect these to be present when menstrual secretion is present, the absence of menstrual secretion markers indicates only blood was present in the sample. This is consistent with the negative log LR given by the model (where the $\beta$’s are the coefficients in Figure 8 and the $r$’s are the marker results in Table 2):

$$\log_{10} LR = \beta_0 + \sum_{i=1}^{p} \beta_i r_i$$

$$= -1.34 + 0.79 \times \frac{3}{4} + (-0.57) \times \frac{4}{4} + (-0.10) \times \frac{4}{4} = -1.4$$

For case 2 all markers that we would expect for vaginal mucosa and/or menstrual secretion are present, the model gives back a higher LR as expected:

$$\log_{10} LR =$$

$$-1.34 + 0.79 \times \frac{3}{4} + (-0.57) \times \frac{4}{4} + (-0.10) \times \frac{4}{4} + 1.45 \times \frac{4}{4} + 2.75 \times \frac{4}{4} + 0.56 \times \frac{4}{4} + 1.35 \times \frac{4}{4} + 2.32 \times \frac{4}{4}$$

$$= 8.5$$

For case 3 again blood is clearly present, but there is a clear indication of vaginal mucosa and/or menstrual secretion, with some expression of the relevant markers. Note that the n/2 method [3] described in the introduction would have concluded “No reliable statement possible”. In contrast, the MLR model output does reflect this indication:

$$\log_{10} LR = -1.34 + 0.79 \times \frac{3}{4} + (-0.57) \times \frac{4}{4} + (-0.10) \times \frac{4}{4} + 1.45 \times \frac{4}{4} + 0.56 \times \frac{4}{4} + 1.35 \times \frac{4}{4} + 2.32 \times \frac{4}{4} = 1.5$$

Knowledge of the case may change this assessment. For example, we know penile skin sometimes gives weak expression on MUC4, MMP10, MMP7 and MMP11 (table 1). Thus, if the sample under consideration were a penile sample, penile skin would form an alternative explanation for the presence of these markers. Thus, we would expect the log LR (for vaginal mucosa and/or menstrual secretion) to be lower. We can compute the log LR taking the penile sampling in account, i.e. by setting the background level for penile skin to 1. This results in adjusted coefficients (fig 8, bottom), and in a log LR of:

$$\log_{10} LR = -1.65 + 0.51 \times \frac{3}{4} + (-0.43) \times \frac{4}{4} + 0 \times \frac{4}{4} + 0.81 \times \frac{4}{4} + 0.82 \times \frac{4}{4} + 0.81 \times \frac{4}{4} + 1.1 \times \frac{4}{4} + 2.4 \times \frac{4}{4} = 0.8$$

which is lower, as expected.
The values found for the coefficients for the model (fig 8) can to some extent be explained. As expected, coefficients are large for markers that were originally selected for their sensitivity to menstrual secretion and vaginal mucosa (fig 8, purple). The coefficient is also large for HBB; this is a marker selected for sensitivity to blood that is expressed for all samples with menstrual secretion measured (see table 1). There are three markers with strong negative coefficients, STATH, BPIFA1 and ALAS2. These markers are expressed in nasal mucosa and blood, which are the body fluids hardest to distinguish from vaginal mucosa and menstrual secretion [12]. Thus, detection of these markers increases the likelihood of the sample containing nasal mucosa and/or blood. Although the presence of these body fluids in itself holds no information on the presence of vaginal mucosa and/or menstrual secretion, it does mean that the presence of vaginal mucosa or menstrual secretion are no longer needed to explain jointly expressed markers (e.g. MUC4). Thus seeing BPIFA1 and MUC4 should yield a lower LR when assessing vaginal mucosa and/or menstrual secretion as body fluid of interest than just seeing MUC4, which is modelled by MLR as a negative coefficient. Note that this is actually a more advanced interaction effect between markers that cannot be modelled by the logistic regression model, which assumes independence. Lastly,
MUC4 and HBB are sometimes detected in penile skin. Thus, in a sample that we know to contain penile skin, the presence of these markers should be less indicative of vaginal mucosa and/or menstrual secretion. This is exactly what we see reflected in the smaller coefficients for these two markers for the model with a penile skin background level of 100% (fig 8 bottom).

Fig 9. Illustration of model predictions using (x-axis) a previously proposed multi-class [15] or (y-axis) the present multi-label logistic regression model, on a sample containing blood, nasal mucosa and vaginal mucosa.

Figure 9 illustrates the advantage of using a multi-label model on mixtures. LRs are plotted for the different body fluids and the combination vaginal mucosa and/or menstrual secretion for a fictitious sample containing blood, nasal mucosa and vaginal mucosa. We constructed the sample to have 'perfect' expression, i.e. all relevant markers (HBB, ALAS2, CD93, STATH, BPIFA1, MUC4, MYOZ1, CYP2B7P1) were expressed for all replicates. This measurement gives strong support for the presence of all three body fluids. As expected, figure 9 shows the multi-label model assigns LR > 1 for each of the three body fluids. However, multi-class logistic regression [15] assigns LR < 1 to menstrual secretion, and LR < 1 to all other body fluids, because by construction it cannot handle mixture data. Note that the presence of blood and vaginal mucosa markers could also have been caused by the presence of menstrual secretion. This makes it hard to say whether vaginal mucosa or menstrual secretion are present, which leads to the multi-label model assigning LRs close to 1 to both. However, it is clear from the markers that at least one of the two body fluids must be present, as is reflected in the high LR (>1000) the model assigns to the combined class of vaginal mucosa and/or nasal mucosa.

Figure 10 gives an overview of conclusions of the n/2 method vs those from the proposed method, for actual casework. 34 out of the 86 traces resulted in a “indication for the presence of vaginal mucosa” reporting using the n/2 interpretation method. Using the developed model, all of these traces resulted in LRs ranging from “moderate support” to “moderately strong support” according to the ENFSI verbal scale (model capped to an LR of 1000 i.e. “moderately strong support”). 23 traces fitted in the category with “no indication for the presence of vaginal mucosa” based on the n/2 method, all except one of these resulted in an LR < 0.5 when the model was used (one sample resulted in an LR of 1.01). The remaining 29 traces fitted the “No reliable statement possible” category. For 16 of these traces, the model resulted
in an LR <0.5. Two traces resulted in an LR between 0.5 and 2 ("do not support one hypothesis over the other") and eight traces in an LR between 2-10 providing "weak support". Three samples resulted in an LR between 10-100 ("moderate support"). Thus for these latter 11 traces, the model provided information supporting the presence of vaginal mucosa and/or menstrual secretion, whereas when using the n/2 method, this information is lost and these traces were reported as "No reliable statement possible".

Fig 10. Overview of conclusion reached for 86 casework traces for (middle column) the n/2 method currently in use and (right column) the proposed logistic regression model.

12 Discussion/future work

We have presented a novel way to probabilistically assess the presence or absence of forensically relevant body fluids, allowing for the presence of multiple body fluids in a sample. We constructed and tested the performance of LR systems based on machine learning models followed by a calibration step. We found that performance, as measured by $C_{llr}$, was similar for different models and multi-label strategies. However, the method seemed more robust when using dichotomized data, showing better performance on a lab-generated mixture dataset, and when using logistic regression, with less sensitivity to deviations from the assumption of equal background levels for the body fluids. Using logistic regression and the one-vs-rest multi-label strategy yields a highly interpretable model, whose predicted $10^{\log LR}$s are a weighted summation of its coefficients. Application to historical casework showed results that were consistent with those given by the currently used ‘n/2’ evaluation method, but more informative for 11/86 cases. We therefore recommend implementing this system in forensic casework, with extra validation studies needed for different lab environments and when body fluids of interest differ from vaginal mucosa and menstrual secretion.

The evaluation of presence/absence of body fluids from mRNA data is difficult as relatively few data are available given the problem complexity. For example, relevant markers like MYOZ1, CYP2B7P1 and MMP7 are seen exactly once or twice for nasal mucosa samples. If all or none of the instances of seeing a marker end up in the training set, any flexible statistical model is likely to over-infer from this that the marker is highly specific. An order of magnitude more data may well be needed to benefit from the flexibility such models offer. This may explain why the previously suggested [15] logistic regression model, often considered a baseline in machine learning literature, performs best. Likewise, more sophisticated multi-label techniques that allow for interactions, such as the here-studied label power set or not-studied classifier chains [38], may only prove their worth when (more) experimental mixture data are available.

To compute LRs in a multi-class setting information on background levels of the different classes is
needed \[15\]. These background levels are not known. In the present study, this information is needed to set the proportion of body fluids in the \textit{in silico} mixtures used to construct the LR systems. There are three reasons we think our assumed background level of 50% probability for the presence of every body fluid, which is unrealistic, is nonetheless acceptable. First, it is a uniform distribution, not giving preference to any of the body fluids. Second, the assumption leads to conservative LR systems. As 50% is probably an overestimate, we obtain more body fluids per sample than would be expected in practice, which is a harder problem for the models to solve. The calibration ensures this leads to LRs closer to 1. Third, the approach allows, for individual cases, to test the impact of different assumptions by explicitly setting the background levels, including the extremes of 0% and 100%.

Although we have focused here on generic, flexible models, better fitting models grounded in biology may be obtained with mathematical modelling of mRNA expression in cells. This would entail describing what mRNA profiles look like as a function of the body fluids present, similar to current practice in forensic DNA analysis. Effects of physiological status, environmental factors and impacts after deposition may have to be taken into account. Although challenging, such modelling would yield several advantages. First, it would increase our understanding of the system, as the data would allow us to test assumptions, and infer key biological properties as model parameters. Second, it may yield a better-performing LR system, both because extrapolations may be feasible and because a post-hoc calibration step would no longer be needed. Third, it would increase our confidence in the resulting LR-system, due to the biological interpretation of the model.

The LR system might further be improved by a more sophisticated handling of menstrual secretion. Biologically, menstrual secretion is a mixture often containing blood and vaginal mucosa, as can be seen from the similarity in markers that are expressed. However, in the current study we ignored this biological knowledge. This could be improved by explicitly modelling the relevant samples as a mixture that can contain blood and vaginal mucosa as well as ‘atomic’ menstrual secretion, which triggers the expression of MMP10, MMP7 and MMP11. In case work, the presence of vaginal mucosa or menstrual secretion often supports the same scenario at activity level, while their absence may support the other. Thus the approach we took here, combining the two body fluids into one set of interest, is a practical way of working around the special status of menstrual secretion. The relation between skin and penile skin may be similar, with penile skin showing a similar pattern of markers to skin but stronger expression of MUC4 (table 1): a marker that is also used for the identification of vaginal mucosa.

Like all empirically based LR systems, the proposed system should not yield very high or very low values of an LR, as these are based on extrapolations that are not supported by the data. If we aim for being maximally conservative, roughly speaking the highest LR supported is the number of independent data points under H2 (see \[39\] for a more sophisticated treatment). Furthermore, as we have seen the calculated value of an LR for a given sample may fluctuate based on assumptions and data used. We therefore recommend to only report the order of magnitude of the LR, possibly using a verbal scale \[40\] and to cap the LR magnitude reported (as in figure 10). The cap depends on the values supported by the size of the dataset, which in our case is in the range of hundreds (max) and 1/hundreds (minimum).

13 Conclusion

We presented a way to construct LR systems for mRNA data that allow for the presence of multiple body fluids, and found that a relatively simple model based on logistic regression performs well, exhibits robustness and is easily interpretable. We recommend using this system to calculate weight of evidence from RNA data to support the forensic expert in case work.

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15 Competing interests

The authors have no competing interests to declare.
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