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HOW FEW POOLED TESTS ARE NEEDED

TO DETECT A SINGLE POSITIVE SAMPLE?

K. GRÆSBØLL*, L.O. ANDRESEN, T. HALASA, N. TOFT

SUMMARY

Testing large quantities of samples to detect one or more positive sample(s) is expensive and time consuming. Pooling of samples can optimize this process. Several different pooling schemes were simulated to compare the efficiency as a function of prevalence and number of pooled samples.

The sensitivity of ELISAs on pooled samples for antibodies in bovine milk to *Salmonella Dublin* (SD), *Mycobacterium avium* spp. *paratuberculosis* (PTB), and bovine virus diarrhea was tested; alongside ELISAs for antibodies in serum to SD, PTB and infectious bovine rhinotracheitis. For milk assays the sensitivity decreased rapidly with increased pool sizes. However, for serum the detection limits were between 25 and 100 individual samples.

The best pooling scheme depended mainly on the prevalence and the sensitivity of the test in a pooled sample. The combinatorial scheme named Shifted Transversal Design proved to be the best framework for determining the most efficient pooling scheme.

INTRODUCTION

Within the veterinary field, pooling is used extensively to detect farm status and/or as an indicator for further investigation. This can be in form of testing the bulk tank milk of dairy cows for, for instance *Salmonella Dublin* (Nielsen et al, 2005), or pooling of ticks, midges or mosquitoes - often in geographical strata (Rasmussen et al, 2013, Lorraine et al, 2014). However, little pooling seems to be have been done when the objective is to detect disease in individual animals.

How few pooled tests are needed to detect a single positive sample? The answer firstly depends on how much a positive sample can be diluted and still be detected; in other words: How does sensitivity change with the pool size? Secondly, how many negative samples does this positive sample hide between; in other words: What is the prevalence?

When determining whether pools are positive or negative, it can be desired to determine alternative lower cut-offs for the ELISAs compared to the defaults determined by the manufacturer. A lower cut-off is needed because pooling dilutes positive samples causing a

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lower signal which lowers the sensitivity of the test. Therefore, alternative cut-offs were determined to as low as possible to increase sensitivity, while maintaining specificity.

Pooling of samples can be done in structures of different dimensionality. The 1D is the traditional pooling method: pool from a line. The 2D is arranging samples in a matrix and pool on the edges. The 3D is to arrange samples in a cube and pool on the edges. Beyond the traditional 123D methods this paper investigates a combinatorial scheme: the Shifted Transversal design (STD), which can also go to even higher dimensions. The advantage of going to high dimensional and combinatorial pooling is that the need to retest samples to identify the individual positive sample may be significantly reduced or even eliminated. The reduction of retests potentially saves time and money. Thus, the objective of this paper is to investigate when testing to determine the individual positive animal; what are the possible savings using pooling; and what is needed to achieve those savings.

MATERIALS AND METHODS

Firstly, pooling experiments with ELISA testing will be presented, followed by the pooling schemes and the approach to simulate them.

ELISA tests

The sensitivity of commercially available ELISAs on pooled samples for detection of antibodies in bovine milk to *Salmonella Dublin* (SD) (PrioCHECK Salmonella AB bovine Dublin, Prionics, Swizerland), *Mycobacterium avium spp. paratuberculosis* (PTB) (ID Screen Paratuberculosis Indirect, IDVet, Grabels, France), and bovine virus diarrhea (BVD) virus (Svanovir BVDV-Ab, Svanova, Uppsala, Sweden) were evaluated using the following approach: The commercially available ELISA tests were performed according to the manufacturer's instructions. The numbers of positive milk samples included in the study were 9 for SD, 8 for PTB and 10 for BVD. Positive milk samples were pooled with known negative milk samples resulting in one positive sample being pooled with 4, 9, 24, 49, 99, 149 and 199 negative samples, respectively. An equal volume from each sample was used for pooling. The optical density (OD) was measured at 450 nm. Results were calculated as percent positivity (PP) by Eq. (1).

$$PP = 100 \cdot (OD_{sample} - OD_{negative\ control}) / (OD_{positive\ control} - OD_{negative\ control}) \quad (1)$$

The sensitivity of ELISAs on pooled samples for detection of bovine antibodies in serum to SD, PTB and bovine herpesvirus-1 (BHV-1) causing bovine rhinotracheitis (IBR) (Nylin et al. 2000) was also investigated in a similar manner. The numbers of positive bovine serum samples included in the study were 7 for SD, 5 for PTB and 4 for IBR. Positive serum samples were diluted in negative bovine serum and tested as undiluted and in dilutions of 1:5, 1:10, 1:25, 1:50, 1:100, 1:150 and 1:200.

Estimation of the specificity of the ELISAs and alternative cut-offs for milk samples was performed by testing 460 known negative milk samples in each of the three ELISAs. The alternative cut-off was calculated as the mean percent positivity relative to the positive control of the assay plus 3 times the standard deviation.

Pooling schemes

The three traditional 123D pooling schemes and the combinatorial STD scheme were tested. The first three schemes have easily identifiable physical representations, while the STD combinatorial scheme requires more complicated pooling and decoding algorithms. For simplicity it is assumed that the test here used has 100% sensitivity and specificity. The pooling schemes are:

1D: This is the traditional pooling scheme in use as far back as 1915 (Hughes-Oliver, 2006). Each sample is pooled once with a number of other samples. If a pool is positive, all samples belonging to this pool must be retested to identify a single positive sample. Notice that in the 1D pooling scheme, there will always be a retest when there are positive samples. This means that a 1D pooling scheme can be expensive if retesting carries a large cost. A 1D pooling scheme is equivalent to testing pools P1-P8 in Fig. 1.

2D: This pooling scheme is also known as the matrix or row/column pooling scheme (Barillot et al, 1991, Hughes-Oliver, 2006). The physical representation of this scheme is all samples arranged in a matrix and then pools are created by sampling all rows and columns. Positive samples can be identified by intersection of positive rows and columns (Fig. 1). Notice that the 2D pooling scheme have situations were retesting is not necessary, most commonly when there is only one positive sample. Therefore, if the pool size is reduced then the probability of retesting will also be reduced.

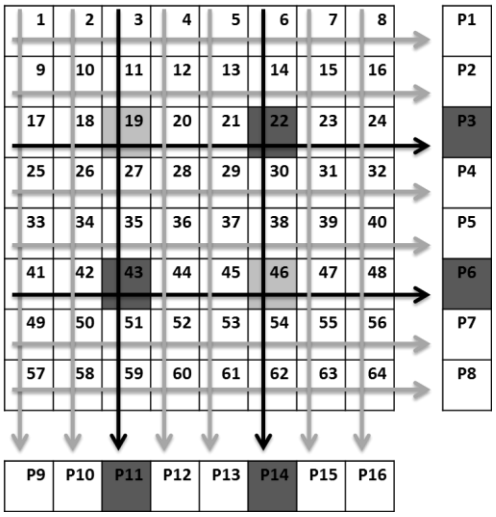

Fig. 1 An example of a 2D pooling scheme with 64 samples pooled into 16 pools of pool size 8. Here samples 22 and 43 are positive, causing pools 3, 6, 11, and 14 to be positive, thus prompting a retest of the samples on the intersections; namely samples 19, 22, 43, and 46.

3D: This pooling scheme is also known as the cube scheme (Barillot et al, 1991). The physical representation is that samples are arranged in a cube (stacked matrices), planes in the xy, xz an yz direction are pooled. Positive samples can be identified by intersection of positive planes (Fig. 2). Because the pools in the 3D are planes of a cube the pool sizes are all square numbers. Therefore, the 3D pooling schemes with pool size 4, 9, 16, 25, and 36 were the only ones examined. Contour plots in the results section of 3D are based on interpolation.

STD: The Shifted Transversal Design was introduced by Thierry-Mieg (2006). It is a combinatorial pooling scheme, i.e. it formalises how to pool samples with a minimal co-

occurrence of samples in the pools – the STD minimizes the amount of times that a given sample is in the same pool as another given sample. Minimizing co-occurrence has the effect, that the STD is a method that can detect multiple positive samples in multiple dimensional pooling schemes - often without retesting. The STD is defined by multiple parameters. However, in this paper results are presented as a function of pool size. There will at most times be more than a hundred different pooling combinations with a similar pool size proposed by the STD, of these the optimal pooling scheme is selected for presentation. For the complete mathematical description of the STD, see Thierry-Mieg (2006).

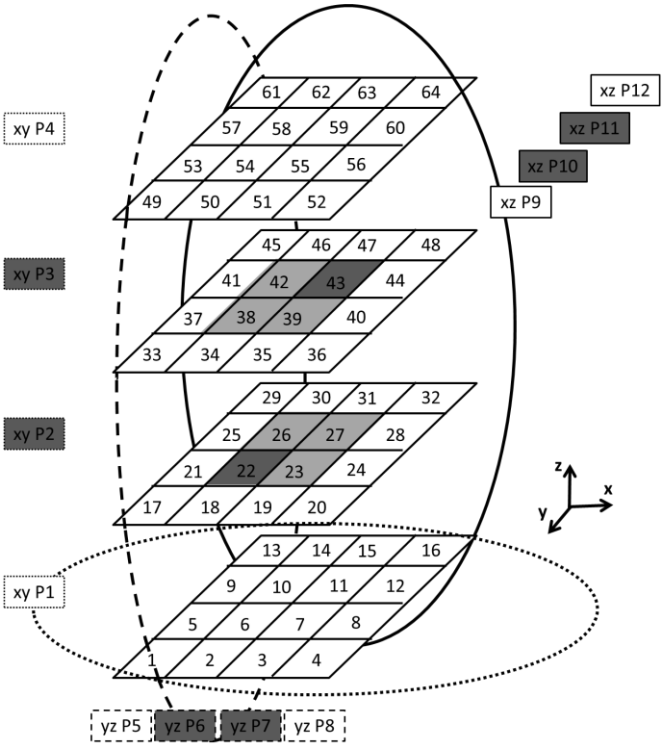

Fig. 2 An example of a 3D pooling scheme where 64 samples are pooled into 12 pools of pool size 16. Pooling is done 4 times along each of the xy, yz, and xz planes. Two positive samples (samples 22 and 43 indicated by dark grey) give rise to 8 possible positives (light grey).

Simulation: For some of the simpler schemes the number of retests can be deduced with relative ease. However, with increasing number of positive samples the combinations of locations of positives in the pooling structures give rise to a very complicated probabilistic structure. For the STD, the number of possible pooling schemes made an analytical solution unfeasible. Therefore, the pooling schemes were simulated to use the average number of retests found in the simulation. The simulation method was: All possible pooling schemes with a pool size smaller than or equal to 36, were tested in combination with prevalences from 0.1% to 90%. For each combination of pool size and prevalence, the number of individual samples that were needed in the scheme was drawn from a binomial distribution. This was repeated a thousand times for each of the 1D, 2D, and 3D schemes and one hundred times for the STD. The number of times to retest and the average number to be retested were saved for each combination of pool size and prevalence. The limit on pool size of 36 was imposed because the time to simulate the STD scheme increases exponentially with pool size. All simulations were done using R: A Language and Environment for Statistical Computing ver. 3.1.1 (R development Core Team, 2014) in RStudio ver. 0.99.447 (RStudio Team, 2015).

Comparison of pooling schemes: When comparing pooling schemes to the testing of individual samples the trivial comparison is to count the total number of tests needed to detect the positive samples. However, there may be costs associated with the pooling itself and/or the storage and preparation for retesting individual samples identified as possible positives by the pooling schemes. In this paper, the cost of pooling is assumed to be negligible, and only a fixed cost for the retrieval of possible positive samples to be retested is included. The cost of testing a single sample or pool is set to index 1, and the cost of retrieving is given as a relative cost to this index. The total cost of a single pooling scheme is number of test used for pooled samples + number of samples to be retested + cost of retrieval. Thus, when the cost of retrieving is set to zero, the costs and the number of tests are equivalent. In the results section, the savings of the pooling scheme are presented relative to the cost of individual testing of all samples. To get savings in Euros, multiply the total number of samples to be tested and the cost of testing an individual sample/pool in Euros onto the values in the savings plots.

RESULTS

Table 1 summarizes the results of the testing of negative milk samples in the three ELISAs. Results from the pooling of the nine SD positive milk samples with negative milk samples are presented in Fig. 3. Similar results were obtained when testing milk samples in assays for antibodies to PTB and BVD virus. Results show that the percent positivity (PP) decreases drastically when positive samples are pooled with increased number of negative samples. Pool sizes higher than 25 showed results at the level of the negative samples. From Fig. 3 it can be seen that a maximum pool size of five would give positive results with the alternative cut-off of 21 PP. Pool sizes higher than five could result in false negative measurements for milk.

Figure 4 shows the results from diluting serum samples positive for PTB in known negative bovine serum. In the case of the ELISA for PTB the positive serum samples could be diluted up to 100 times, corresponding to a pool size of 100, and still have a positive result in the ELISA using the cut-off of 70% positivity. For SD and IBR positive serum samples, the maximum pool size was 50 and 10, respectively, when using the default cut-off values recommended by the manufacturer.

Table 1. Results from test of negative milk samples in ELISA

ELISA for antibodies to*	Negative samples tested	Mean Percent Positivity	Standard deviation	Alternative cut-off	Default cut-off	Specificity using alternative cut-off
Salmonella Dublin	460	6.65	4.62	21	35	0.99
Mycobacterium avium spp. paratuberculosis	460	2.00	2.18	9	15	0.995
Bovine Diarrhea Virus	460	3.50	0.93	7	12	0.99

*ELISAs used are presented in materials and methods

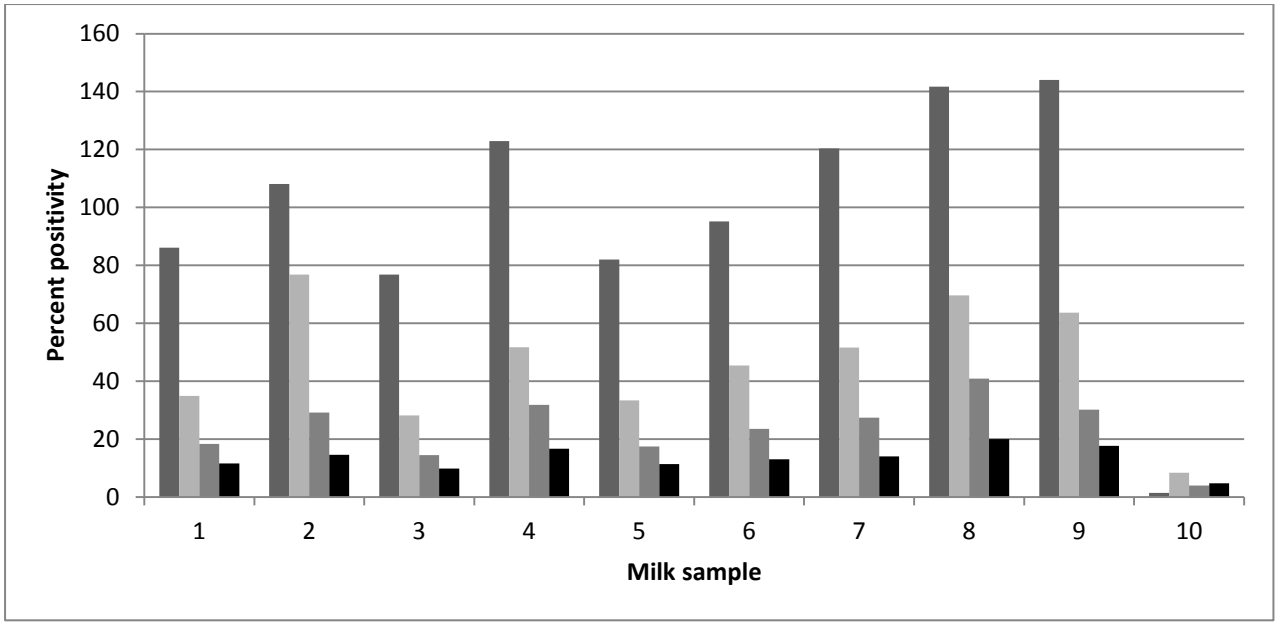

Fig. 3 Salmonella Dublin ELISA: Percent positivity in pooled milk samples. Nine antibody positive samples and one antibody negative sample were pooled with known negative milk samples. Results of testing the milk samples undiluted (dark grey), diluted 1:5 (light grey), 1:10 (grey) and 1:25 (black) are presented as mean values of three tests performed on separate days.

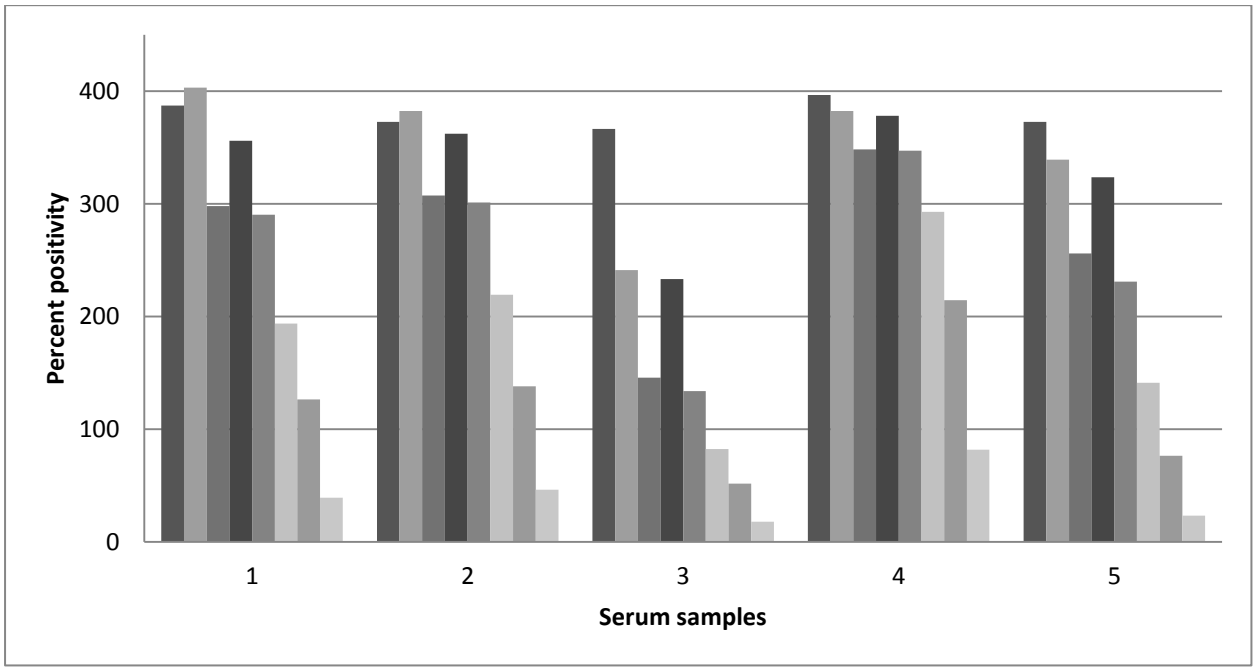

Fig. 4 PTB ELISA: Percent positivity in pooled serum samples. Five antibody positive samples were diluted in known negative bovine serum. Bars from left to right represent results for undiluted serum, and serum dilutions 1:5, 1:10, 1:25, 1:50, 1:100, 1:150 and 1:200, respectively.

The results of the simulations show that STD can provide the most efficient pooling schemes regardless of pooling size or prevalence (Figures 5, 6, and 7). It can be difficult to interpret what goes on in the STD, for that reason 123D are compared, because the

mechanisms that govern the optimal schemes within those schemes generally also apply to the optimal scheme selected by the STD.

Figures 5 and 6 show that a 1D scheme is more efficient than 2D and 3D when the maximum pool size, the retrieval cost and the prevalence are low. However, as soon as the retrieval cost increases, 1D is less often the optimal scheme. Generally, for higher retrieval cost the pooling schemes of higher dimensions becomes optimal.

There exists for all pooling schemes an optimal pool size for a given prevalence (dashed grey lines in Figures 5-7). It can also be observed that pooling is not cost-efficient when the prevalence is higher than 30% (Fig. 5). If only the maximum prevalence is known (Fig. 7), there is a large difference between the 1D and the higher dimensional pooling schemes. The 1D scheme is only cost-efficient if the maximum prevalence is below 10%, while the 2D can be cost-efficient for a maximum prevalence as high as 50%.

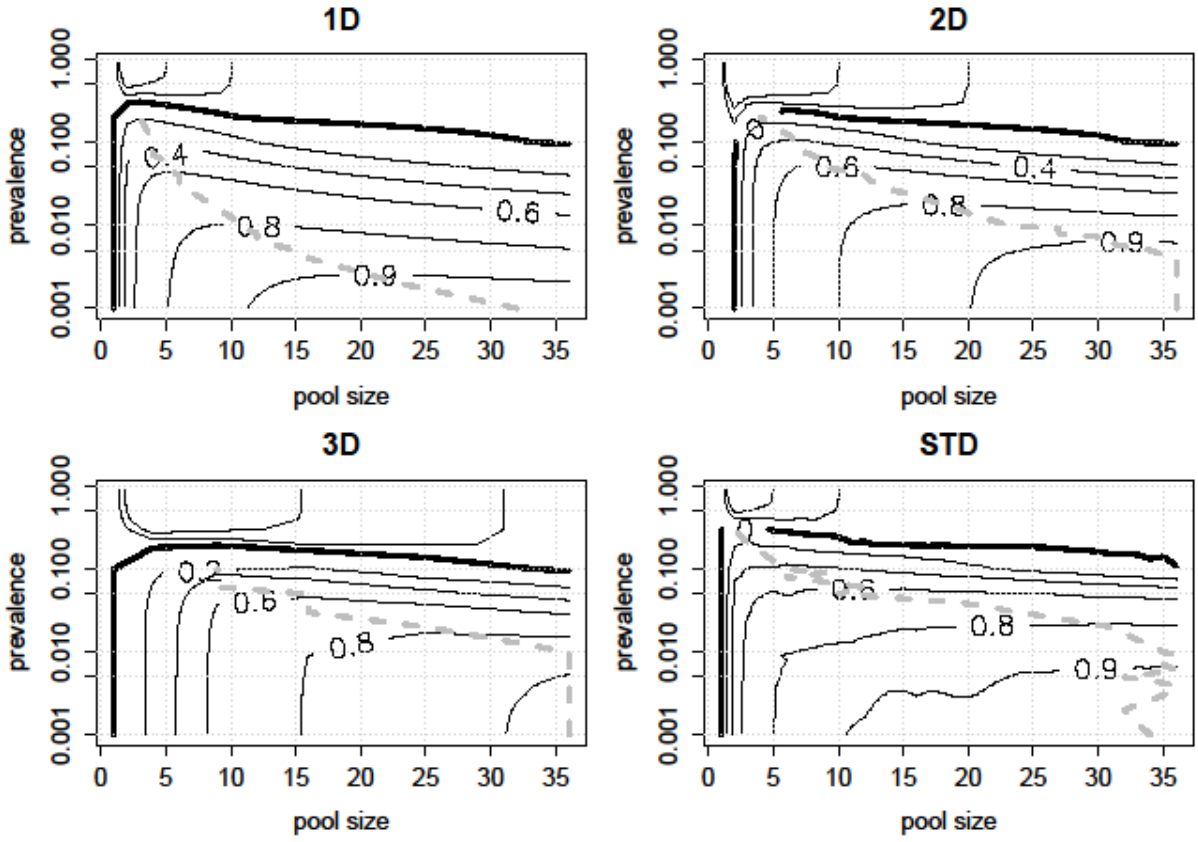

Fig. 5 Contour plot of the fraction of tests saved compared to individual testing as a function of pool size and prevalence. The thick line indicates where the pooling scheme requires the same number of tests as individual testing, and above this line pooling cannot be cost effective. However, there are large areas in the bottom right corners where the number of tests saved is more than 50%. The grey line indicates the pool size that gives the maximal saving of tests for a given prevalence. This plot is equivalent to a savings plot where the cost of retrieval is 0.

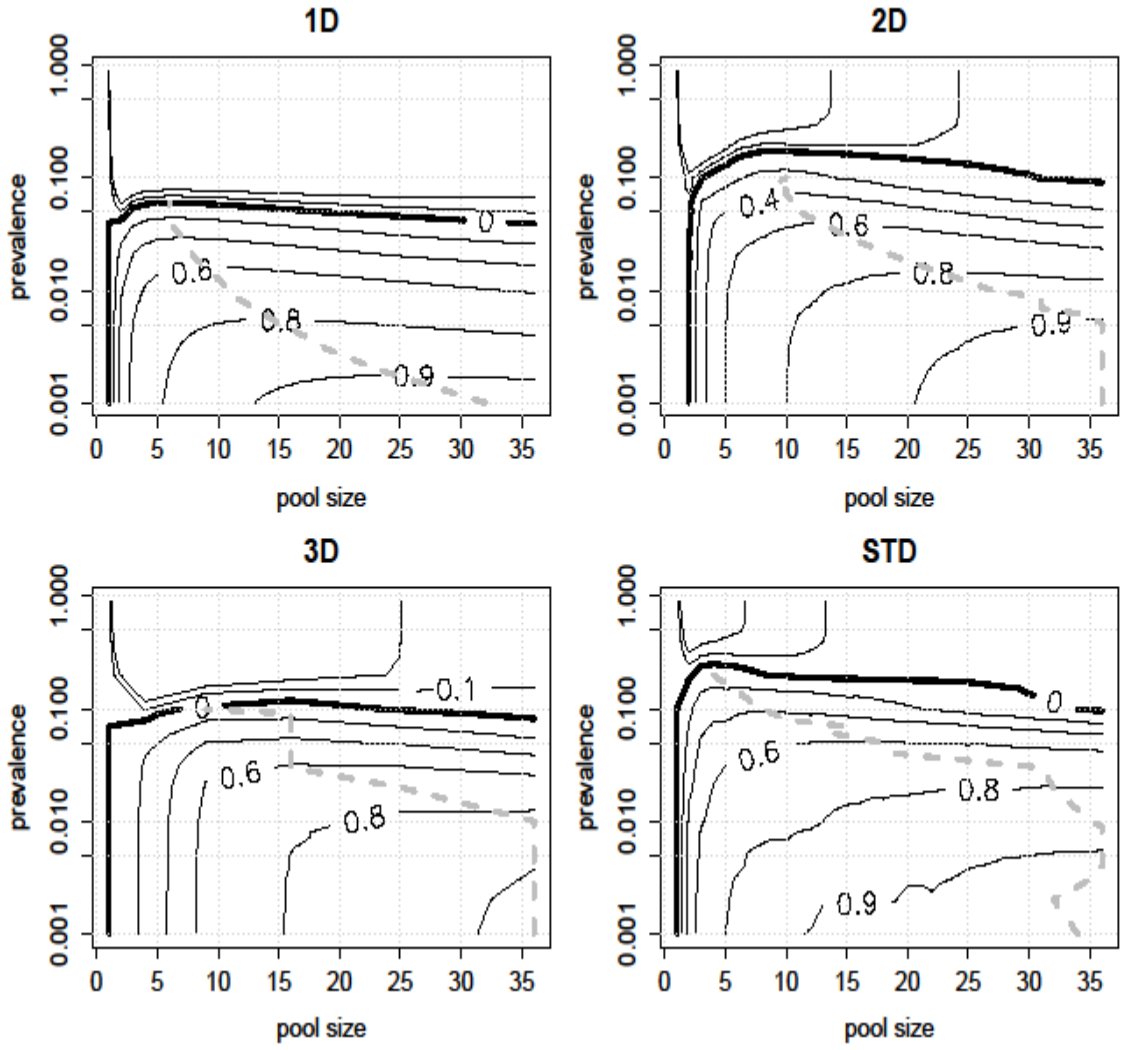

Fig. 6 Contour plot of the fraction of savings compared to the price of individual testing as a function of pool size and prevalence, where each retrieval for retesting caries the cost of 10 individual tests. The thick line indicates where the pooling scheme has the same cost as individual testing, and above this line pooling is not cost effective. However, there are large areas in the bottom right corners where the savings are more than 50% compared to the price of individual testing. The grey line indicates the pool size that gives the maximal saving for a given prevalence.

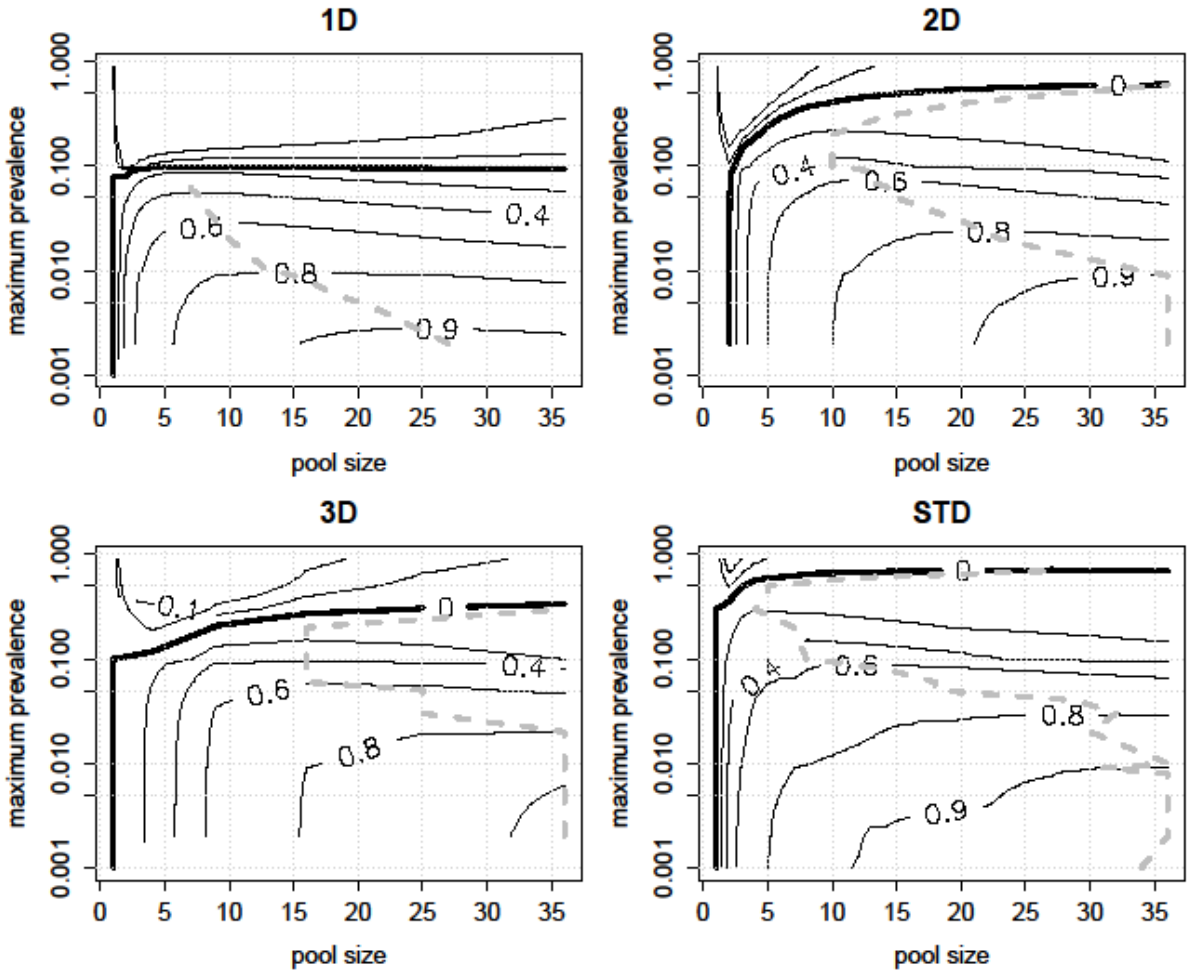

Fig. 7 Contour plot of the fraction of savings compared to the price of individual testing as a function of pool size, but only the maximum prevalence is known. Prevalence is assumed to be uniformly probable between 0.001 and the maximum prevalence. Every retrieval for retesting caries the cost of 10 individual tests. The thick line indicates where the pooling scheme has the same cost as individual testing. The grey line indicates the pool size that gives the maximum saving for a given maximum prevalence.

DISCUSSION

The physical representations of some of the pooling schemes are not necessarily connected to the ideal way of implementing them in practise. The 1D and 2D schemes can be done by conventional pipetting, e.g. 'by hand', but higher dimensional pooling schemes and the STD are more easily done by a robot. The robot should not arrange samples in the physical structures, but rather from software receive a list of pools that each sample is assigned to. In this way, each individual sample is only visited once, and directly distributed to the relevant number of pools determined by the pooling scheme. In this paper the additional cost of pooling were assumed to be zero, because large scale laboratories often have a robot that distribute samples from test tubes to test assay (personal communication with Eurofins Steins Laboratory). For 1D pooling this robot could deliver the sample to a pooled well on the assay instead of individual wells without increasing time spend. For higher dimensional pooling

schemes each sample need be delivered to multiple wells, this cost in time may be negligible if e.g. the cleaning process between samples or the time to test is comparatively longer.

The sensitivity and specificity were not specifically handled in the simulations. For a specific disease, dilution series to determine Se/Sp for different pool sizes should be performed. The results in this paper are reported in terms of the pool size, which may allow a user to impose his or her cut-off in OD to the desired Se/Sp. To use the results in such a way, it must be assumed that Se/Sp is sample specific: A sample that test negative in an individual test will also test negative in all pooled test, and vice versa. Furthermore, it has been assumed that samples do not give rise to added unspecific reactivity when pooled.

The maximum pool size for a given disease using a specific test kit can be determined by dilution trials as presented in this paper. In this work, this initial step was further used to determine an alternative cut-off in order to maximize the possible maximum pool size. The experiments presented here are examples. To achieve a good measurement of the change in sensitivity when pooling the number of positive samples tested should be larger. Specifically, weakly positive samples must be included in the test series, to correctly estimate changes in sensitivity due to pooling. It may also be possible to adjust the procedure of the ELISA if there are steps of pre-dilution before the OD measurements to further increase sensitivity (Brinkhof et al, 2007).

In this paper only positive/negative test results following the use of a cut-off are reported from the pooling schemes. However, using cut-offs can remove information from the test, and it could potentially be better and/or easier to make algorithms to identify positive samples based on the distribution of the continuous outcomes in a pooling scenario.

Hierarchical group testing is a class of pooling schemes that require additional number of retests as samples are pooled, and re-pooled depending of the results of the first pools (Black et al, 2015). Preliminary results show that hierarchical schemes can be cost optimal if price of retesting is low.

The results indicate that a higher retrieval cost leads to pooling schemes of higher dimensions becoming optimal. This is due to higher dimension schemes contain more samples per scheme, and given retrieval is a onetime expense, this will lower total cost.

The STD is always the most cost effective scheme, because it implicitly also includes the 123D schemes and many more. The difference between the STD and the other schemes does, however, depend on the price of retesting and the combination of pool size and prevalence. Within the explored parameters in this paper, the STD was seldom more than 10% points better than any of the other schemes. However, in Thierry-Mieg (2006) it was shown that for very low prevalence combined with opportunity to go to high pool sizes the STD becomes very efficient compared to simpler schemes.

CONCLUSION

The results of the simulations show that for a wide range of prevalences and pool sizes, there are large potential savings with pooling. However, certain restrictions apply before those savings can be achieved: Firstly, initial tests must be performed on the specific test kit intended for use, to determine how large a pool size can be used, ideally in combination with defining an alternative cut-off to maximize Se/Sp. Secondly, samples should not display an increase in any unspecific reactivity in the test when pooled, otherwise results may be invalid.

The code that produced the savings figures in this paper has been integrated into a Shiny WebApp, which allows the user to specify the cost of retrieval and the cost of pooling. The WebApp is freely available at https://kagr.shinyapps.io/SMARTPOOL.

ACKNOWLEDGEMENTS

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