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A method for improved fluorescent staining for acid fast smear microscopy by incorporating an acetone rinse step

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ABSTRACT

Microscopic examination of the specimen smear for acid fast bacilli (AFB) provides a simple and rapid means of detecting AFB using fluorescent stain methods and remains a valuable diagnostic test used worldwide to identify and manage suspect cases of tuberculosis (TB). Methods to improve AFB smear staining protocols could provide better detection of suspect TB cases. In particular, decreasing background debris may improve the detection of smears with low numbers of bacilli. We assessed staining by the standard rack method compared to bulk container staining using an acetone rinse step to decrease background debris. No cross-contamination was observed in the bulk container staining, and higher accuracy with less reading time was achieved with the acetone rinse. Most importantly, more bacilli were detected per positive smear using the acetone rinse method.

1. Introduction

Despite the routine use of molecular detection methods for *Mycobacterium tuberculosis* (Mtb), the microscopic examination of the smear for acid fast bacilli (AFB) provides a simple and rapid means of detecting AFB, with fluorescent staining methods considered the most sensitive (Hendry et al., 2009; Steingart et al., 2006). In low-income, high-burden countries, it is often the only diagnostic test available to identify cases of tuberculosis (TB), and in high-income, resourced countries, the AFB smear determines the next steps in the laboratory testing algorithm and is used by TB control programs for case management (Rea and Rivest, 2014). It is important for laboratories to ensure that they are proficient in AFB smear preparation and microscopy, and methods to improve AFB smear staining protocols can provide better detection of suspect TB cases (Pfyffer, 2015; Somoskövi et al., 2001). Ideally, these method improvements should facilitate safe, accurate, high-throughput processing while mitigating risks of error and cross-contamination.

The ability to accurately detect low concentrations of bacilli is partially dependent on the absence of background debris. While the species of mycobacteria cannot be determined by AFB smear microscopy, *Mycobacterium avium* complex isolates are significantly smaller bacilli than Mtb and can be difficult to detect in AFB smears (Atlas and Snyder, 2015). Acetone rinsing has been demonstrated to reduce background interference from debris but cannot be used on slide racks in the open laboratory due to risks associated with solvent vapors (Burke and Dunning, 1924). These risks can be reduced by moving rack staining procedures into a fume hood; however, the impact on throughput would be significant. Alternatively, bulk acetone rinsing in containers with lids would permit use in the open laboratory to increase throughput but could potentially increase the risk of cross-contamination (Kam et al., 2009).

In this study, we assess the performance of an in-house–developed bulk container staining method incorporating an acetone rinse step compared to the standard auramine O/rhodamine B (AR) fluorescent staining method using individual slide racks(Truant et al., 1962). Prior to evaluating the overall performance of the acetone-rinse staining protocol and as a key performance characteristic, the potential increased risk of cross-contamination from batch processing by using bulk staining in containers was first verified that it did not result in cross-contamination.

To ensure maximum fidelity to smears observed in routine clinical laboratory conditions, all materials used were derived from positive and negative clinical specimens. Performance was measured by calculating the time required by staff to stain and read multiple prepared slides as well as the percentage of Mtb- and *M. avium* (Mav)–positive smears correctly identified by each method.

2. Materials and methods

2.1. Slide preparation

Low positive (1+ smears;1–9 AFB/10 fields at 200×) (Public Health Agency of Canada, 2014) of Mtb and Mav were prepared from a

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resuspended pellet of specimen concentrate according to the in-house protocol used for quality control and proficiency program slide preparation. Briefly, 4+ (>100 AFB/field at 200×) sputum specimens received at PHOL from known Mtb culture positive patients were pooled, and an equal volume of 5% phenol was added to inactivate the organisms. Similarly, negative specimen material (>20 pooled smear and culture negative specimens) and smear-positive 4+ specimens from known Mav culture-positive patients were also prepared (*M. avium* isolates identified using the GenoType Mycobacterium CM line probe assay (Hain Lifescience, Nehren, Germany). All smear load assessments as described above (4+ Mtb/Mav and negative) were originally performed on patient specimens using the individual slide rack method (Truant et al., 1962). The material was then centrifuged, and a volume of supernatant was removed to bring the specimen material to the original pooled volume. Aliquots from the negative specimen material were then titrated to 1+ by the addition of the Mtb and Mav prepared material (Aziz et al., 2002). Ten paired sets of 3 slides (custom-coated 6-well slides, product #30-228H-Black, Erie Scientific LLC, Portsmouth, NH) containing 36 smears in each pair were then prepared simultaneously from the above thoroughly mixed low positive and remaining negative specimen material, and all slides were heat-fixed on a heat plate at 140 °C until dry. Slides were then inactivated by flooding (rack) or immersion (glass staining dishes) in 5% phenolic alcohol for 5 min. Each paired slide set was prepared to contain 2 groups of 3 slides with the same number of overall Mav and Mtb 1+ positive smears in randomized slide well locations. Each set of 3 slides would undergo the differential staining methods as described below. Ten participating technologists performed this staining independently for each prepared paired set. Slides were stored in a closed slide box, away from light. Each of these technologists was not involved in the preparation of the smear material or the smears and was blinded to the location of the positive smears on each slide.

2.2. Slide preparation — bulk container staining cross-contamination verification

New microscope slides were cleaned by wiping with a 70% ethanol soaked Wypall pad (Kimberley-Clark, Mississauga, Ontario, Canada) and dried prior to use. Smears were prepared from clinical sputum specimen material remaining from previously tested specimens. Ten sets of slides were prepared, each consisting of twelve 4+ positive smears and 12 negative smears, from specimens of both “thick” and “thin” consistency to reflect the variability observed in purulent versus aqueous specimen sources (e.g., sputum versus bronchoscopy wash). Each set contained smears from specimens positive for Mtb, Mav, and *M. abscessus* (Mabs) to represent large, small, and medium-sized bacilli, respectively.

2.3. Slide staining — acetone rinse verification

For nonacetone rinse individual slide rack staining, heat-fixed and inactivated slides were processed according to manufacturer's recommendations: Slides were flooded with AR (Auramine O > 80%, Rhodamine B > 95% HPLC, Sigma-Aldrich Canada Co., Oakville, Ontario, Canada) for 15 min and then briefly rinsed in running tap water. Slides were decolorized by flooding with acid alcohol (0.5% hydrochloric acid in ethanol) for 30 s, which was poured off and followed by a repeat flooding with acid alcohol for 2–3 min. Slides were rinsed in running tap water and flooded in 0.5% potassium permanganate (Public Health Ontario Laboratory, Toronto, Ontario, Canada) for 4–5 min followed by a brief rinse with running tap water.

For the bulk container staining with the acetone rinse, heat-fixed and inactivated slides were immersed in a glass rectangular staining dish (DWK Life Sciences, item# 900203, Rockwood, TN) containing AR stain for 9 min followed by a running tap water rinse in a dish. Slides were then immersed in the first acid alcohol containing dish for 30 s, followed by immersion in a second acid-alcohol–containing dish for 30 s, and then rinsed in a third dish with running tap water. The slides were then immersed in the first acetone containing dish (reagent grade acetone [99.5%], Caledon Chemicals, Georgetown, Ontario, Canada) for 30 s (lid closed) followed by immersion in a second acetone containing dish for 30 s (lid closed). Slides were then immersed in a potassium permanganate containing dish for 45 s followed by a brief rinse in another dish with running tap water. Reagents in the staining dishes were changed, and the staining dishes were cleaned as follows: AR stain weekly, acid alcohol and acetone when discoloration appears, and potassium permanganate twice weekly.

2.4. Slide staining — bulk container cross-contamination verification

All 10 sets of slides were passed through the staining dishes as per the bulk container acetone rinse staining method in succession as described above. None of the reagent containing dishes were refreshed or topped up for the cross-contamination verification. Positive smears were positioned in the staining dish rack to face negative smears, and the arrangement of slides containing Mav/Mtb/Mabs and thick/thin slides was randomly placed in the rack. At the completion of staining the slide sets, 2 “blank” slides (no smear) were passed through the same reagent containing dishes. Slides were read independently by 3 experienced TB laboratory technologists, and results were compared.

2.5. Slide reading — acetone rinse verification

Each participating technologist read a group of 3 slides stained by each method. The slides were then passed on to 2 different technologists, who were blinded to the staining method used for each group of slides within the set. Each technologist recorded the grading and the subjective quality of the smear (fluorescence, background) using a rating scale from 1 (poor, highly fluorescing background material with low bacillary fluorescence) to 10 (excellent, clean black background with intensely fluorescing bacilli). The time required to read each group of smears was also recorded. Each of the 10 sets containing 18 smears stained by each method (total of 540 smear observations per method) was thus read and evaluated by 3 different technologists (Fig. 1). Each of these technologists routinely prepares and reads smears in the clinical laboratory.

3. Results

The comparison of average preparation and staining time, subjective quality rating, and proportion of correctly identified smears that were prepared with Mtb or Mav between the standard rack method and bulk container acetone rinse method is shown in Table 1. Less hands-on time was required for the bulk container staining with acetone rinse method compared to standard rack staining. Slides stained by the bulk container acetone rinse method scored higher in subjective quality rating, were stained and read more rapidly, and yielded more smears identified as positives for both Mtb and Mav. Additionally, more smears were rated as ≥1+ using the bulk container acetone rinse staining method than the individual rack staining method despite all positive smears being prepared to the same 1+ standard as shown in Table 2. Overall, there were net decreases between the individual rack and bulk container staining methods in the number of smears assessed as negative and “Few” with net gains in the number of smears assessed as 1+ and 2+. Individual rack staining and bulk container staining without the acetone rinse step were also compared and showed no significant differences in subjective quality score, time to read, or percent positive observed. There were also no significant differences between results when technologists read slides stained by themselves or others (data not shown). Slides were read the same day they were stained by the staining technologist and within 2 weeks by the other technologists. A small number of slides could not be read due to faded fluorescence

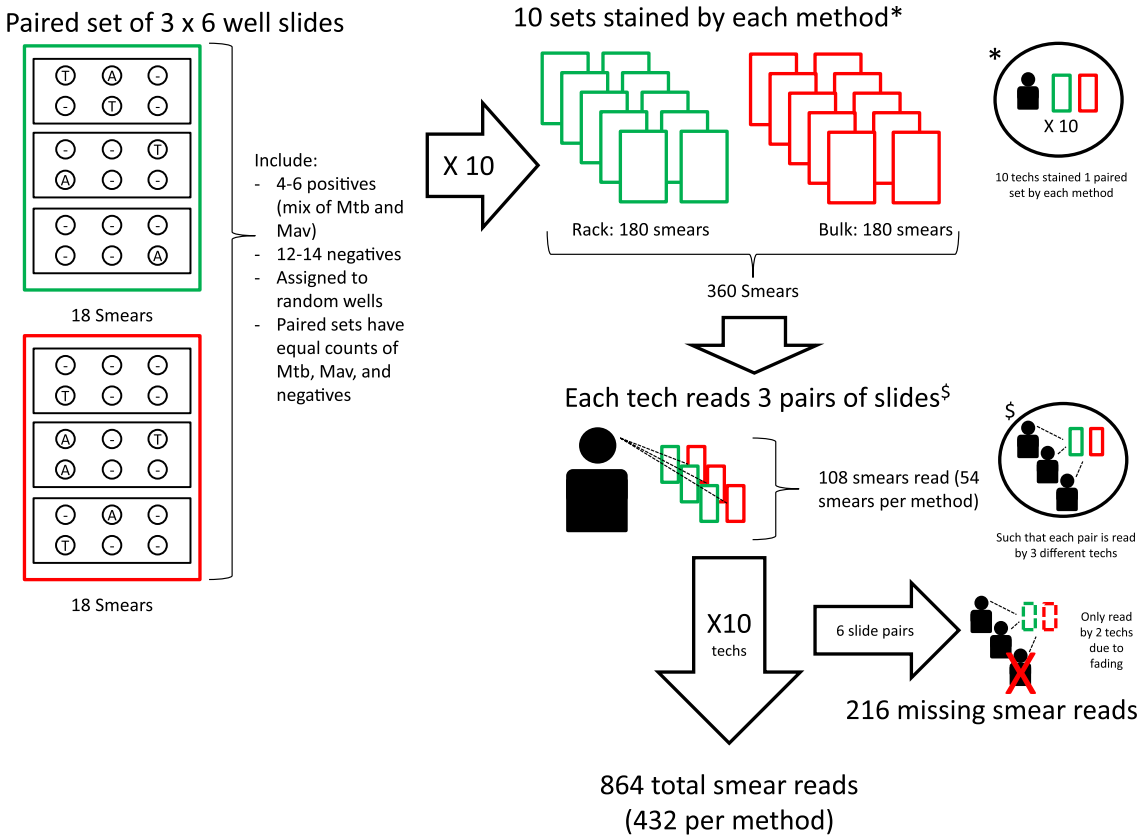

Fig. 1. Diagrammatic representation of bulk staining acetone rinse step method smear reading by technologists. "A" = *M. avium*, "T" = *M. tuberculosis*.

caused by unavoidable delays greater than 2 weeks between staining and reading (not associated with the study design). This reduced the total number of observations to 432 per method from 540 (Fig. 1).

The cross-contamination verification of the bulk container acetone rinse staining method provided no evidence of positive material transfer between slides. None of the negative slides facing the 240 positive smears passed through the serial reagent container baths were found to show evidence of cross-contamination. Additionally, the 2 blank slides passed through the baths following staining of all positive slide sets remained negative.

4. Discussion

This study was performed in a large public health laboratory (Public Health Ontario Laboratory) setting that provides tuberculosis and mycobacteriology diagnostic and reference testing services. Over 50,000 specimens are processed annually, with over 200 smears routinely read per day. Improvements to the staining time and quality of the stained smear are important components of laboratory efficiency and can have significant operational impacts in this setting (Hendry et al., 2009). Advantages of bulk container staining include reduced workload and resource requirements, and the ability to utilize automated staining instruments (Kam et al., 2009). Decreasing background fluorescence has been previously shown to improve the quality of reading slides (Clancey et al., 1976; Hendry et al., 2009). Specifically, the use of acetone in the decolorization step of the Ziehl–Neelsen staining method described by Burke et al. provided significant reduction in background signal (Burke and Dunning, 1924). In our study, slides stained with acetone were consistently rated of better quality with less background debris and more intensely fluorescing bacilli, and were read more quickly by experienced technologists (Fig. 2). More Mav positives were also correctly identified in less time on average using the bulk container acetone rinse staining method. This finding is significant given that Mav bacilli are typically smaller and more susceptible to masking from background fluorescence than Mtb, and in Ontario, a larger proportion of specimen smears contain Mav due to increasing rates of Mav disease (Marras et al., 2013). Better method performance was also reinforced with an increased number of positive smears rated as ≥1+ using the bulk container acetone rinse step staining method compared to the individual rack staining method. Despite

Table 1
Comparison of fluorescent acid fast staining by standard individual rack and bulk container with acetone rinse methods.*a, $b

Performance metric	Individual rack staining	Bulk container with acetone rinse staining	Acetone improvement
Average subjective quality rating[a]	6.5	8.2	N/A
Time to stain 18 smears (min)	27	17	37.0%
Time to read 18 smears (min)	16.7	14.8	11.3%
Percent of Mav found	80.3 (53/66)	90.9 (60/66)	10.6%
Percent Mav rated ≥1+[b]	42.4 (28/66)	57.6 (38/66)	15.2%
Percent of Mtb found	94.0 (47/50)	96.0 (48/50)	2.0%
Percent Mtb rated ≥1+	34.0 (17/50)	54.0 (27/50)	20.0%

[a] Subjective quality rating scale from 1 (poor) to 10 (excellent).
[b] 1+ rating is equivalent to 1–9 acid fast bacilli per 10 fields at 200× magnification.

Table 2
Smear grade by staining method and organism for 360 prepared smears (264 negative, 42 Mtb, 54 Mav) read by 3 different technologists[a] (n = 864 reads).*a, $b

Smear rating[b]	Individual rack staining reads (Mtb/Mav)	Bulk container with acetone rinse staining reads (Mtb/Mav)	Net change from individual rack to container with acetone
Negative	330	322	−8
Few	29/25	21/21	−8/−4
1+	17/23	22/27	+5/+4
2+	1/5	5/12	+4/+7
False positive	2	2	0

[a] Six pairs of slides (216 smears composed of 160 negatives, 26 Mtb, and 30 Mav) were unable to be read by the third technologist due to fluorescence fading and were excluded. Positive smears were prepared to a standard 1+ rating.

[b] "Few" grade is equivalent to 3–9 AFB/entire smear at 200× magnification, 1+ grade is equivalent to 1–9 acid fast bacilli per 10 fields at 200× magnification, and 2+ grade is equivalent to 1–9 acid fast bacilli per field at 200× magnification.

all positive slides being prepared to approximately the same +1 standard, the decreased background fluorescence allowed a greater proportion of them to be correctly and more easily identified as 1+ or greater, rather than identified as negative or "few" (3–9 AFB/entire smear at 200× magnification). Increased proficiency in reading low bacillary load ("Few" or 1+) smears is particularly important in high-volume settings such as in our laboratory since they require more time to read and can represent a significant proportion of smears. In our laboratory, 2253 of 60,491 smears read (3.7%) were rated as "Few" or 1+ versus 1662 smears (2.7%) rated 2+ or greater (2016 data, unpublished). Although the percentage of Mtb positives detected was only marginally improved using the bulk container acetone rinse staining method, the commensurate 20.0% increase in smears rated ≥1 + using this method helps underscore the increased ease with which low bacillary load Mtb smears could be read. Taken together, the increased performance and decreased time for staining and reading the AFB smear using this method have the potential to positively impact and improve efficiency and accuracy of smear microscopy in the mycobacteriology laboratory.

The primary limitation of the bulk container acetone rinse staining method relates to solvent fumes from the acetone, and the impact that mitigation of this hazard has on processing throughput and cross-contamination risk associated with the need to use bulk containers for

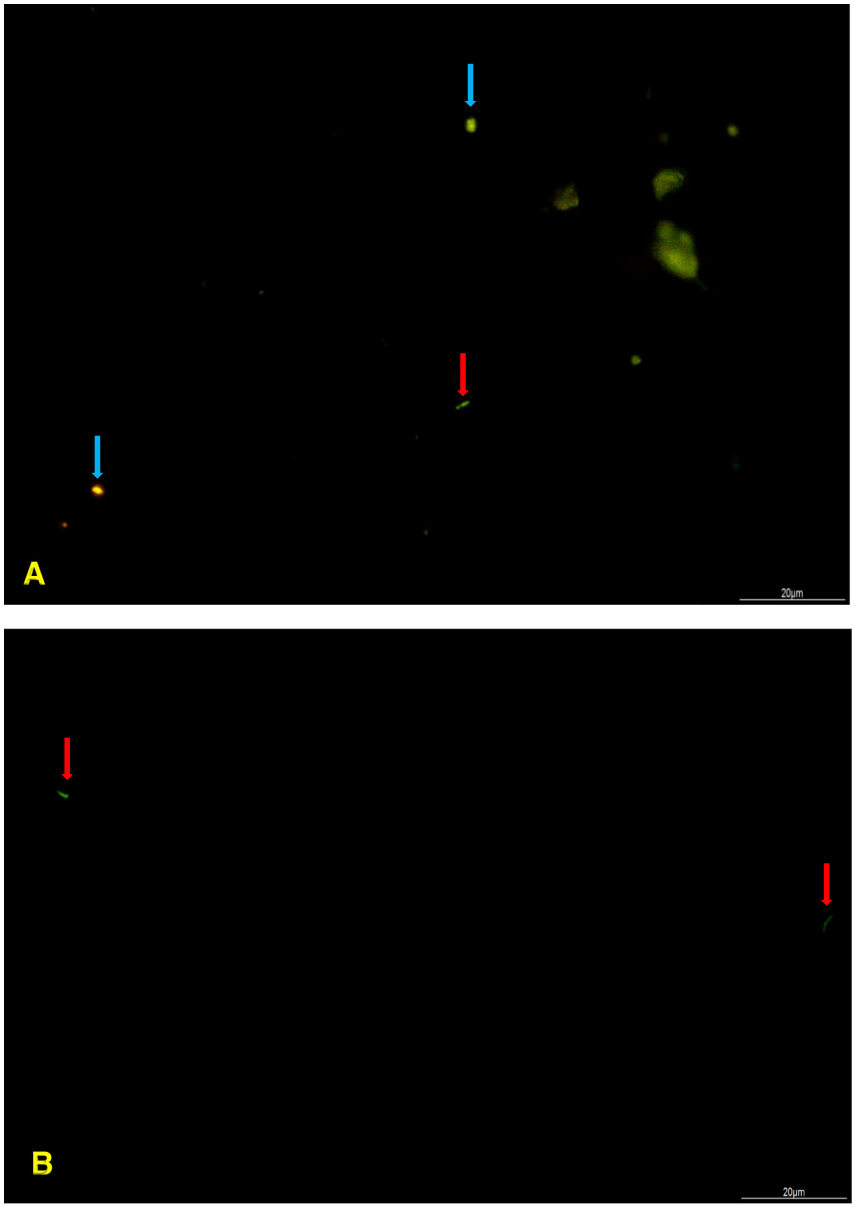

Fig. 2. Rack AFB stain method, 600× (A) and acetone stain method 600× (B). Red arrows indicate bacilli; blue arrows indicate some examples of debris. 2+ smear.

staining. Solvent fumes are best captured by enclosed fume hoods which usually have a small footprint and are not compatible with the large open surface areas required for staining slides on individual racks. Bulk container staining can effectively reduce the bench space footprint sufficiently to allow manipulations with acetone in a fume hood and has been previously shown to improve both the quality and consistency of staining (Affolabi et al., 2008; Burke and Dunning, 1924; Clancey et al., 1976). Bulk container staining has however remained controversial due to a perceived increased risk for cross-contamination despite previous studies failing to find evidence to confirm this risk (Affolabi et al., 2008; Kam et al., 2009). Similarly, our results found that bulk container staining provides a more efficient, less laborious, and simpler means to prepare large numbers of slides for AFB microscopy while mitigating risks associated with solvent vapor. Significant time savings were achieved in the staining process (27 vs. 17 min on average), and the addition of acetone which bulk container staining permitted yielded higher-quality results as described above. Moreover, we confirmed that placing large numbers of positive smears (equivalent to 3 months of 4+ smears from the routine clinical workload in our laboratory) through reagent bulk containers without changing the reagents did not result in transfer of positive material to adjacent or subsequent negative slides.

Potential limitations to this study include that materials prepared from negative smears (known culture negative specimens) may have contained false smear negatives, thus skewing towards increased sensitivity. We felt that the appearance fidelity of clinical specimen–derived negative smears (compared to artificial sputum matrix) outweighed this risk. Additionally, the performance improvements observed on prepared standardized slides may not be completely representative of those obtained on clinical specimens prepared under routine operating conditions. Following this study, the bulk container acetone rinse staining method was implemented for routine clinical diagnostic testing in the TB and mycobacteriology laboratory at the Public Health Ontario Laboratory in 2014 and has been in use since then. There has been no change observed in the number of specimens submitted to our laboratory, and adoption of the bulk container acetone rinse staining method has resulted in a significant increase in detection of smear-positive specimens in 2014–2015 relative to the previous period (2011–2013) for which data were available (Table 3, Poisson regression, IRR = 1.52, 95% CI = 1.45–1.59, *P* value <0.001). Additionally, there was no increase in the number of smear-positive, culture-negative specimens, which suggests that the increase in smear positives was not a result of "overcalling" the smear (725 of 4770 [15.2%] for 2011–2013 versus 797 of 5184 [15.4%] for 2014–2015). Underlying changes in submitter specimen collection practices and/or disease epidemiology however cannot be ruled out as contributing factors to this increase. Additionally, no evidence of cross-contamination events has been identified in the routine quality control slides included in all staining runs with the implementation of the bulk container acetone rinse staining method, and the laboratory has not incurred any external proficiency testing program errors for AFB smears. Laboratories considering implementing the bulk container acetone rinse staining method should ensure that control slides are included in every slide run. Taken together, our results indicate that the bulk container acetone rinse staining method for AFB fluorescence smear microscopy demonstrated a significant improvement over the conventional rack staining method in terms of accuracy and throughput with no impact on laboratory safety and no evidence of cross-contamination.

Table 3
Total *Mycobacterium tuberculosis* isolations and proportion smear positive by year and smear preparation method, 2011–2015, Public Health Ontario Laboratory, Ontario, Canada.

Year	Method	Total Mtb	Smear positive (percent)
2011	Rack, no acetone	1112	596 (53.6)
2012	Rack, no acetone	1066	526 (49.5)
2013	Rack, no acetone	1245	652 (52.4)
2014	Bulk acetone	1170	684 (58.5)
2015	Bulk acetone	1169	699 (59.8)

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