

Microwave assisted silver stain for pap smear to improve the identification of coccobacillary flora

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Abstract

Introduction: Pap smear has been utilized since a long time in detecting cervical pre-cancers and cancers. Their potential in diagnosing pathogenic organisms however is underestimated. Reporting organisms morphologically consistent with *Candida* species, in cervicovaginal smears, is easier, but coexisting coccobacillary flora is often missed in the bargain. Such co-existing infections make the treatment difficult for clinicians, prolonging the distressing symptoms of patients. We evaluated modified silver staining technique using a microwave - JONES MARRES STAIN (JM) and tried comparing the results with conventional Pap smears to find any additional flora.

Aim: To identify co-existing organisms in addition to *Candida* in a Pap smear stained with Jones Marres stain

Materials and methods: This retrospective prevalence study that included 82 consecutive conventional Pap smears routinely stained with Pap stain was then subjected to silver stain in a microwave.

Inclusion criteria: Pap smears reported as showing *Candida* group of organisms

Results: In the 82 smears evaluated, JM stain helped in easier picking up of additional coccobacillary flora in 37(45%) of cases in comparison to 4(4.87%). These coexisting infections were missed in conventional Pap smear reporting.

Conclusion: Modified JM stain helped in diagnosing additional flora thus reducing the chances of missing out co-infections and hence is useful for providing effective treatment

Keywords: Microwave Silver stain, Co-infection, Pap smear, Coccobacillary flora.

Introduction

The use of Pap smear for reporting pre-cancers and cancers of uterine cervix dates back to as early as 1940s.¹ But its role in diagnosing inflammatory pathologies has grossly been underestimated since its advent. In a country like India where inflammatory pathologies hugely outnumber the malignant pathologies, the Pap smear can help in the precise identification of the type of cervico-vaginal flora. The vaginal microflora was first described by Doderlein in the year 1922.² Lactobacilli (Doderlein's bacilli) are the commensals of the vaginal tract and release Hydrogen peroxide, lactic acid and other antimicrobial factors by release of glycogen from (cytolysis of) intermediate squamous cells. This renders the pH of the vagina acidic.³ Widely studied, bacterial vaginosis, is a very common polymicrobial infection, and is generally associated with a shift towards alkaline pH.⁴

Gardnerella vaginalis was thought to be the only organism associated with bacterial vaginosis (BV) but with time it was found out that it actually is a mixed anaerobic infection.⁵ The smears of women with BV show squamous epithelial cells covered with coccobacilli, the so-called 'clue cells'.⁶ Lactobacilli are notably absent.⁷ Inflammatory cells may or may not be seen. Dual pathology in the form of Candidiasis and bacterial vaginosis is a well-known entity.⁸ Such cases of coinfection form the chunk of 'difficult to treat' patients. The Pap smear, a commonly done test in all symptomatic and asymptomatic women, serves to be an easy, fast and highly sensitive modality for suggesting a diagnosis of both.⁹

However, the utility of the Pap smear in reporting 'shift in flora' is limited either by the deterring inflammatory background much found in association with *Candida* infections or when the coccobacilli are few in number and the shift is partial. Also distinguishing the background protein muck from tiny dust like bacteria is sometimes very difficult.¹⁰ Hence, there arises the need of some special stain that can be applied to the routine Pap smear and that would help accentuate the visibility of these microbes and make their identification easy.

The Silver staining techniques have long been used to stain fungi in brains of immunocompromised hosts.¹¹ The same technique holds relevance in staining the various flora in cervicovaginal smears as well. For the latter, a rapid microwave technique, the Jones Marres (JM) silver stain technique, has been suggested.¹⁰ The additional advantage of the rapid microwave technique is that, the 'turnaround time' is very less as compared to the usual silver staining techniques and also that the stain can be done without removing the Papanicolaou stain. Our research question was, whether or not there is additional flora and fauna in a Pap smear reported as showing fungal organisms morphologically consistent with *Candida* species. We conducted this study with the aim to identify, additional cervico-vaginal flora or coexisting organisms, in addition to *Candida*, in a previously stained Pap smear, using the modified JM stain.

Materials and methods

This was a retrospective prevalence study done for a period of 3 months and total 82 consecutive conventional

Pap smears reported as, benign cellular changes of inflammation showing fungal organisms morphologically consistent with *Candida* species, were included. These smears were sent from our Gynaecology outpatient department and were collected irrespective of the presence or absence of symptoms of cervicitis or vaginitis. The slides were reported by a single observer.

Method of JM Stain: Following the reporting by Pap stain, the slides were subjected to silver staining technique, the Jones Marres stain, without being destained.¹⁰ Silver stains are best suited for withstanding high temperatures, a microwave offers.

In an attempt to standardize this stain in our set up and with the settings of our microwave we had to modify the JM stain. We observed that solutions like Gomori's silver stain and periodic acid would boil when subjected to higher temperatures of the microwave for a longer duration. Therefore to adapt to this, we used a power of 450 Watt when using the Periodic acid solution and it was kept at 500W when staining with Gomori's silver stain. The slides were placed in (0.5%) Periodic acid solution and kept into the microwave at 450 Watt and 40 degree Celsius for two minutes. After that they were again kept in the microwave at 100 Watt and 50 degree Celsius for 3 minutes. The slides were then rinsed in distilled water and were subjected to Gomori's Methanamine Silver for 4 minutes at 85 degree Celsius at 500 watt. The slides were again rinsed with distilled water and stained with Gold Chloride (0.2%) for 1 min. They were then rinsed and washed in distilled water and Sodium Thiosulphate for 1 minute respectively. Slides were again kept in the microwave with Periodic acid at 45° Celsius and 450 watt for 2.5 minutes. Then they were finally washed with distilled water and counterstained with Eosin. The Lactobacilli, fungi, coccobacillary flora and any other

bacteria stained brown black. This stark contrast produced by the stain helped in easy identification of the cervico vaginal flora.

Results

Our study included 82 Pap smears reported as 'benign cellular changes (BCCI) associated with *Candida* group of organisms'. In these smears we also looked for the presence of other pathogens in the form of coccobacillary flora or any other flora including the commensal lactobacilli. Of the 82 candida positive Pap stained smears, 4 smears were reported as having coccobacillary flora and 3 showed other flora in addition to candida, in the form of spores and delicate bacilli and very short rods. These 82 Pap smears were then subjected to modified JM silver stain without destaining or removing the Papanicolaou stain. All the 82 smears stained with the modified JM stain showed candida either as filamentous form (fig. 1a) or budding yeasts (fig. 1b). With the modified JM stain the *Candida* appeared 'brown black' and other flora also stained similarly there by making it easy to visualize them. The flora that was previously poorly visualised, could now be easily seen. (fig. 1c)

Keeping *Candida* as the common variable in all Pap smears, the results were tabulated into three categories (Table 1) shows results of Pap smears before and after the modified JM stain. With the modified JM stain, the pickup of the coccobacillary flora (fig. 1d) increased from 4 cases to 37 cases (fig. 2) which was a substantial rise and the p values were significant (p<0.001). The number of smears showing other additional non-classifiable flora also rose (from 3 to 4), however the p values were not significant.

Table 1: Results of Pap smear before and after JM Stain

	Report on conventional PAP smears		Report on Jones Marres stain		p value
	Number	Percentage	Number	Percentage	
Candida only	75	91.46%	41	50%	<0.001
Candida and Coccobacillary flora	4	4.87%	37	45.12%	<0.001
Candida and other flora	3	3.65%	4	4.87%	<0.721

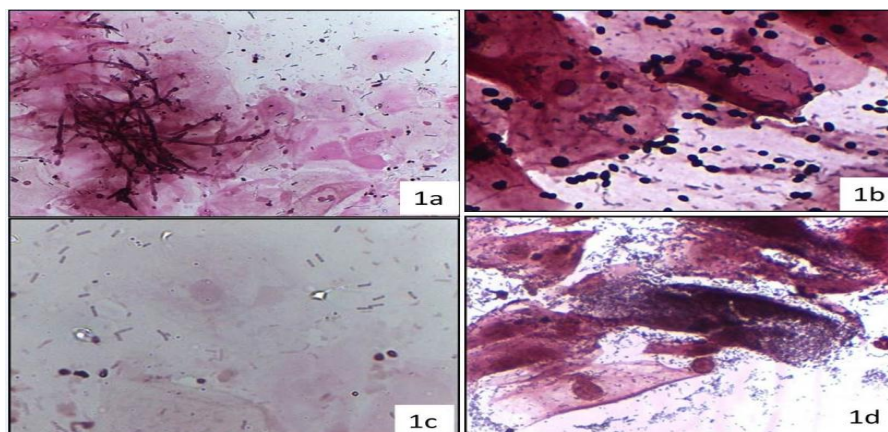


Fig. 1:

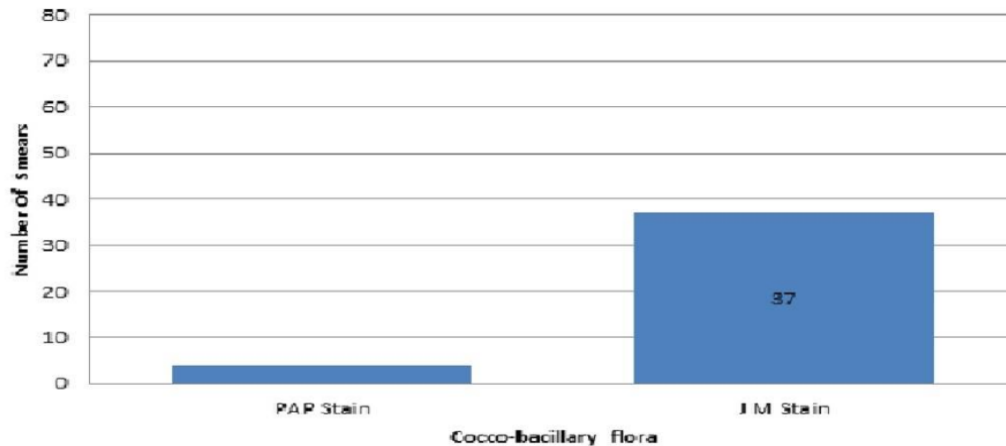


Fig. 2:

Discussion

The normal physiological inhabitants of the vagina are the rod-shaped Lactobacilli. They are responsible for the low vaginal pH (3.5 ± 4.5) as they produce lactic acid from glycogen,¹² The generation of hydrogen peroxide (H_2O_2) by lactobacillus represents a non-specific antimicrobial defence mechanism of the physiological vaginal ecosystem.¹³ However, not all women have lactobacillary flora alone. In some instances, candida (commensal), short (rods) or bacilli, cocci (round) or coccobacilli may coexist in varying proportions or one of these may predominate. When these pathogenic microbes flourish there occurs a simultaneous decrease in the commensal bacteria of the vaginal microbiota.¹⁴ As a consequence, clinical diagnosis must rely on methods that identify proportions of bacterial morphotypes in the vaginal specimen.¹⁵

Women with shift in flora towards coccobacillary type and presenting with clinical symptoms are diagnosed as having Bacterial Vaginosis. Bacterial vaginosis (BV) is one of the most common lower genital tract conditions in the reproductive age group contributing to more than 60% of all vulvovaginal infections. Around 15% to 30% of pregnant women,¹⁶ and 5% to 15% of women attending Gynaecology clinics are diagnosed as having bacterial vaginosis. Of importance is that, BV may be associated with an increased risk of acquisition of HIV,¹⁷ serious pregnancy complications, including premature rupture of the membranes, preterm delivery,¹⁸ postpartum endometritis,¹⁹ and pelvic inflammatory disease. Such potentially hazardous outcomes of BV mandate the need for an accurate and clearly understood diagnosis.

The diagnosis of BV is problematic,²⁰ because the organisms classically associated with BV, i.e. Gardnerella vaginalis, can be recovered on laboratory media not only in symptomatic women, but also in 36 to 55% of asymptomatic women.²¹ In addition, culture and identification of other 'look alike' bacteria such as, Bacteroides species, Peptostreptococcus species and Mycoplasma hominis has been evaluated and found to be specific but insensitive and costly to the laboratory.²² Other anaerobic bacteria strongly associated with BV, such as

Mobiluncus species, are very difficult to recover by culture.²³ When these pathogenic microbes flourish there occurs a simultaneous decrease in the commensal lactobacillary flora.

Various diagnostic tests, though subjective, like the Whiff test, Gram's stain with Nugents score and culture studies, are indicative of BV.²⁴ Nugents score is considered to be the gold standard.²⁵ However, it is a tedious and time consuming technique and requires an experienced reporter.²⁶ These microbiological techniques are limited in their predictive values as they identify only few species of organisms from the various causative agents of bacterial vaginosis.²⁷ The Pap smear has an added advantage over culture that the flora and fauna can be observed in their natural habitat and in their original proportions.¹⁰ In the conventional Papanicolaou stain, the Haematoxylin component binds with the DNA of the micro-organisms, and the Eosin binds with other proteins.¹⁰ Depending on the pH values of the staining dishes, the micro-organisms will stain blue (mainly Haematoxylin), purplish (Haematoxylin and Eosin) or reddish (mainly Eosin). In any of these cases, the amount of dye which is bound to the micro-organisms is small and the contrast very low. Moreover, it is often very difficult to distinguish small bacteria from background particles. Hence it would be of great interest to use a staining method in which their visibility is enhanced by binding to a dye with a contrasting colour. Silver staining methods provide an easily available option. Also, the method is simple, fast and able to be performed on routine Papanicolaou-stained smears.

In this study, we subjected the Pap smears to a silver stain that utilizes the microwave technique. The modified microwave technique of Jones Marres stain was helpful in picking up more flora, in addition to that which was reported by the conventional Pap stain. There is a mention of the typing of flora into 4 classes according to the silver stain: Type 1 - predominance of lactobacilli with no coccobacilli; Type 2 - the lactobacillary flora is much lower than in type 1 but the lactobacilli still predominate with presence of limited number of coccobacilli; Type 3-mixed

flora like mixture of lactobacilli with various other short, plump or round bacteria including the comma shaped Mobiluncus, Streptococci etc, along with few 'clue cells'; and the Type 4-cocoid or coccobacillary overgrowth with abundance of cocoid bacteria in the background, plenty of clue cells and no lactobacilli.¹⁰ Type 2 and 3 smears are usually the ones which escape the eyes of the diagnosticians as the number of coccobacillary flora is limited and the commensal flora is also variably present. When evaluating inflammatory Pap smear, infection with various morphotypes of Candida group of organisms is said to correlate well with inflammatory cells but coccobacillary flora usually do not incite noticeable inflammatory response, thereby making a comprehensive diagnosis all the more tricky.²⁸ Hence the supporting evidence in every smear in the form of inflammatory cells is very misleading.

In the study of Moghaddam et al, in silver stained slides, there was a clear relationship between the type of vaginal microbial flora (other than lactobacilli) and the presence of Candida species.²⁹ They demonstrated a statistically significant association between the microbial flora in type 1 ($p=0.02$) and type 4 smears ($p=0.003$). On similar lines we could also find an increased coexistence of candida and various coccobacillary flora after staining with the modified technique of JM stain ($p<0.001$).

In the study of Boon et al,¹⁰ because of the ease of visualisation of organisms that they achieved following the JM stain, they could demonstrate in the first place a statistically significant relationship between Candida and Actinomyces species. Whereas our focus in this study was to find out the number of Pap smears having the presence of shift in flora along with the Candida morphotypes. We too observed that there was an increase in the visualisation of coccobacillary flora following JM stain and therefore better pick up rates leading to identification of greater number of smears showing co infection by candida and coccobacillary flora ($p<0.001$). Secondly, Boon et al studied the association of these two organisms in the various types of microbial flora and found that Actinomyces coexisted maximally with the mixed type i.e. type 3 of microbial flora (72%) and candida coexisted when there was a predominance of lactobacilli i.e. type 1 situation (37%). In our study we did not attempt to type the flora into various classes as done by Boon et al.

In the study by Mendoza et al done in Mexico, making use of another special stain for Pap smear (Gram's Stain), they studied the association between various vaginal microbes and observed that candida existed maximally with bacterial vaginosis.³⁰ Our study corroborates with the above findings with difference being in the special stain used, which in our study was modified JM stain.

The patient in whom co infection is missed adds to the increasing pool of non-responding vulvovaginitis cases that are actually harbouring dual or polymicrobial infectious agents. A special stain like JM or Gram's stain helps in accentuating the visibility of the delicate coccobacilli which can otherwise go unnoticed especially when the shift is partial. This brings a huge impact from treatment point of

view in prescribing the right kind of vaginal wash to the patient in addition to the conventional antimicrobial treatment. Acidic vaginal washes, when added to the treatment protocol can help in quick reversion to the lactobacillary flora.³¹ Probiotics being live organisms are quick to change the pH of the vagina. Even Yoghurt, household vinegar, as vaginal washes are known to promote the growth of lactobacilli thereby normalising the vaginal pH (to acidic) and curtailing the growth of Coccobacillary flora.

On the contrary, alkaline washes in the form of Soda Bicarbonate are prescribed to treat chronic Candidiasis.³² Recurrent candida infections are difficult to cure using antifungal agents alone.³³ Adhesion of the spores of candida is inversely proportional to the pH of the vagina,³⁴ and therefore additional therapy in the form of alkaline washes scores fairly well in treating recurrences and chronic infections. The presence of candida in a Pap smear conveniently overshadows the associated coccobacillary flora by virtue of the intense inflammatory response that it incites.²⁸

An inflammatory condition like bacterial vaginosis can also lead to great dilemmas when choosing the treatment modalities. The formation of a resistant biofilm by some strains of Gardnerella vaginalis have made the strains more robust and resistant to known modalities of treatment.³⁵ Usually an empirical therapy incorporating Metronidazole, Ciprofloxacin and Clindamycin are the baseline drugs to treat polymicrobial infections, is offered to the patients. Supportive treatment with acidic and alkaline douches has proven effective in giving quick and prolonged relief to the patient.³⁶

Conclusion

Co-existing coccobacillary flora when present along with Candida, is often missed on routine Pap smears, and hence can be a cause of chronicity of vaginal discharge. Precise identification of both can help to prescribe antimicrobial and specific adjuvant therapy for bacterial vaginosis. This remarkable jump in the diagnostic capabilities of the Pap smear would be possible if it is empowered by special stain like the modified JM stain. Modified technique of the JM stain helps in easy identification of cervicovaginal flora by providing better contrast and therefore enhanced visualisation. Point worth mentioning, to conclude our findings is that, though Pap smear is usually considered to be of utility for reporting dysplasia and malignancy, its effectivity in diagnosing inflammatory pathologies is no less and it's sensitivity can be increased by adjunctive staining techniques described in this paper.

Conflicts of Interest: None.

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