

Enzymatic assay to test diamines produced by vaginal bacteria

Kevin Mendonca¹, Caterina Costa¹, Valentina Ricci², Gianni Pozzi¹

¹Laboratorio di Microbiologia Molecolare e Biotecnologia (LAMMB), Dipartimento di Biotecnologie Mediche, Università di Siena, Italy;

²MICROBIOTEC srl, Toscana Life Sciences, Siena, Italy

SUMMARY

An enzymatic assay was developed to determine the concentration of diamines (DA) in clinical samples of vaginal fluids. Putrescine and cadaverine are DA produced by anaerobic bacteria and are typically present in the vaginal fluids of women with an abnormal microbiota, as occurs in bacterial vaginosis. The vaginal DA (VADA) assay is based on the enzyme diamine oxidase which reacts with putrescine and cadaverine to produce H₂O₂ in a quantitative manner. H₂O₂ concentration is measured spectrophotometrically by a chromogenic reaction catalyzed by horseradish peroxidase. The VADA assay proved to be capable of detecting DA concentrations as low as 4 µM and showed a dose-response relationship which was linear over DA concentrations ranging from 4 to 256 µM. Using clinical samples it was possible to show that the VADA assay can be performed on human vaginal swabs and that the mean DA concentration is significantly higher in samples positive for microbial pathogens.

KEY WORDS: Putrescine, Cadaverine, Diamines, Vaginal microbiota, Dysbiosis, Bacterial vaginosis.

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Diamines (DA) putrescine and cadaverine are small aliphatic hydrocarbon molecules produced by anaerobic bacteria during the catabolism of basic amino acids. It is widely acknowledged that polyamines, including DA, are important factors involved in multiple aspects of pathogenesis, survival and virulence of many human microbial pathogens (Igarashi *et al.*, 1974; Dela Vega *et al.*, 1996; Patchett *et al.*, 1996; Cohen, 1997; Jung *et al.*, 2003). Diamines are present in the vaginal fluids of women with bacterial vaginosis (BV) where they can be detected by the so called “whiff” test, in which a characteristic malodour is developed when KOH is added to vaginal fluids containing DA (Amsel *et al.*, 1983). BV is a common condition

in women of child-bearing age characterized by an abnormal vaginal microbiota (dysbiosis) (Nugent *et al.*, 1991; Eschenbach, 1993; Hillier, 1993; Ma *et al.*, 2012). Elevated amounts of DA such as putrescine and cadaverine in the vaginal fluid have been demonstrated to be a typical feature of women with vaginal dysbiosis, while samples of vaginal fluids from healthy women do not contain these amines (Cook *et al.*, 1992, Wolrath, 2001). To investigate the possible use of DA as biomarkers for evaluating vaginal health, we developed a simple enzymatic assay which can be used to quantify vaginal DA in clinical samples, and can be easily adapted to the clinical laboratory setting:

Reagents

Stock solutions of putrescine and cadaverine were prepared in distilled water and kept at 4°C for a maximum of 7 days. The colour developing reagent was prepared fresh before use by mixing four parts of 1.5M Tris buffer pH 9.0, with one part of 400 mM 4-aminoantipyrine, and one part of 40 mM phenol. A frozen stock

Corresponding author

Gianni Pozzi, LA.M.M.B

Dipartimento di Biotecnologie Mediche

Università di Siena, Policlinico Le Scotte

V lotto piano 1

Viale Bracci - 53100 Siena, Italy

E-mail: gianni.pozzi@unisi.it

of diamine oxidase at 1600 milliunits/ml in water was thawed at room temperature just before use. The stock solution of horseradish peroxidase was prepared at 175 units/ml in water and kept at 4°C for a maximum of 15 days. All Reagents were purchased from Sigma-Aldrich Co.

VADA Assay

The vaginal diamine (VADA) assay is based on diamine oxidase which produces H_2O_2 upon reaction with DA like putrescine and cadaverine (Tabor, 1951). H_2O_2 reacts with horseradish peroxidase in the presence of 4-aminoantipyrine and phenol to produce the chromogen quinoneimine (maximum absorbance at 510 nm) in a quantitative manner. The VADA assay was performed on 96-well polystyrene microtitre plates (Nunc, 25361). Samples were added to the wells in a 0.1 ml volume, and always run in duplicate. The colour developing reagent (45 μ l), horseradish peroxidase (5 μ l), and DAO (50 μ l) were then added in sequence. The plate was incubated at 50°C for 1 hour. Optical density (OD) was read with a spectrophotometer set at a wavelength of 510 nm, peak of maximum absorbance of quinoneimine. Clinical samples were tested both with and without addition of DAO to check for the possible presence of H_2O_2 produced by lactobacilli of the vaginal microbiota or by human cells such as macrophages. For each sample the OD value obtained without addition of DAO was subtracted from that obtained with DAO. The resulting OD was used to calculate the concentration of diamines using as a reference a standard curve obtained with a mixture of cadaverine and putrescine at 1:1 molar ratio (range 4-256 μ M). Results were expressed as μ M of DA per sample.

Clinical Samples

Vaginal swabs from 65 women of child-bearing age undergoing routine microbiological analysis at the Bacteriology Unit of the Siena University Hospital were used in this study. The dry swabs were suspended in 1 ml of sterile physiological saline and then subject to microbiological analysis and to the VADA assay. Aerobic and anaerobic pathogens as well as of lactobacilli were detected by standard culture-based methods.

The performance of the VADA assay was tested

on both putrescine (Figure 1A) and cadaverine (Figure 1B) using serial dilutions both in water and in pooled vaginal samples. The detection limit of the assay was 4 μ M for both putrescine and cadaverine, whereas the dose-response relationship resulted linear over a range of DA concentrations varying from 4 to 256 μ M, with R values <0.99 for the regression lines (Figure 1). There was no difference between the performance of the VADA assay in water and in pooled vaginal samples, indicating that clinical samples from the human vagina are unlikely to contain factors capable of interfering with the assay. Since the assay cannot distinguish between the 2 DA, the standard curve for the VADA assay was prepared using a mixture of

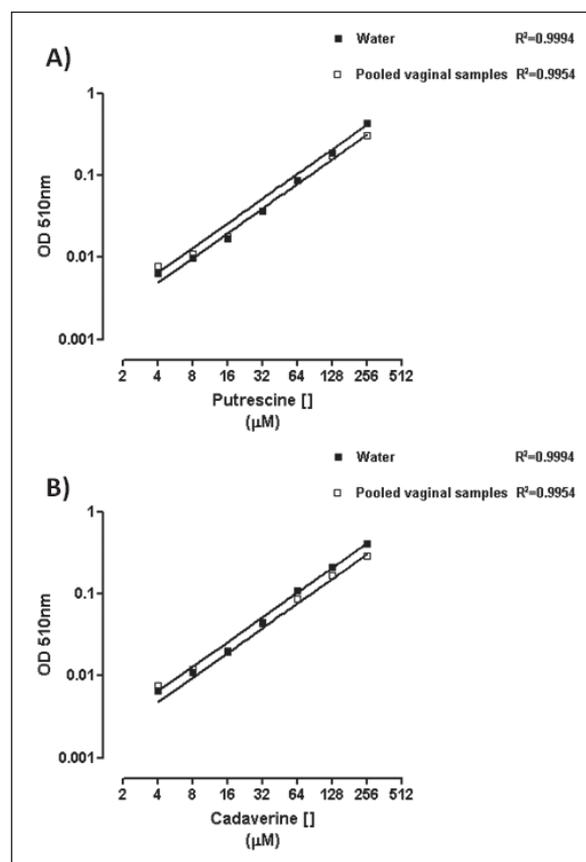


FIGURE 1 - Performance of the VADA enzymatic assay on serial dilutions of putrescine (A) and cadaverine (B). Diluents used were water (filled squares) and a pool of vaginal samples (open squares). Coefficients of the regression lines (R^2) were in all cases >0.99. Concentrations of DA of 2 mM resulted below the detection limit of the VADA assay.

cadaverine and putrescine at a 1:1 molar ratio. As an initial attempt to verify the use of the VADA assay, 65 vaginal swabs were tested for the presence of DA. DA were detected in 29 out of 65 vaginal swabs, with concentrations ranging from 4 to 142 μM of DA per sample. Preliminary data obtained correlating DA concentration in the 29 DA-positive samples and results of the microbiological analysis indicated that the mean concentration of diamines in samples with bacterial pathogens, was significantly higher than in samples without pathogens (Fig-

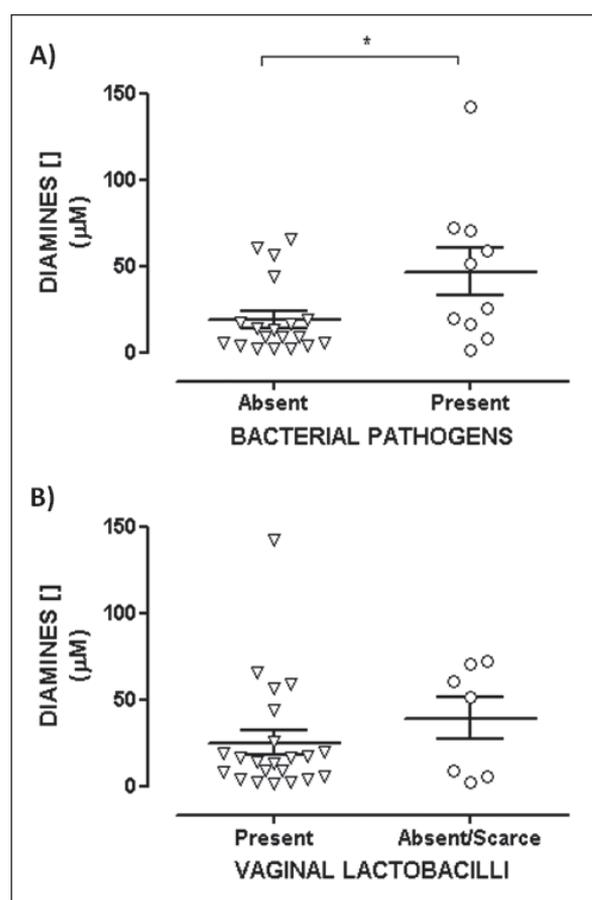


FIGURE 2 - Correlation between DA concentration and presence of microbial pathogens (A), and presence of lactobacilli (B) in the 29 DA-positive vaginal samples. The mean concentration of DA in samples with microbial pathogens (46.7 ± 13.4 mM) was significantly higher ($P=0.012$, one-tailed unpaired *t*-test) than that in samples without pathogens (18.8 ± 4.8 mM), while the difference in the mean concentration of DA of samples with or without lactobacilli was not statistically significant ($P=0.116$, one-tailed unpaired *t*-test).

ure 2A), while the presence of lactobacilli did not seem to correlate with DA concentration (Figure 2B).

Methods to detect putrescine and cadaverine in vaginal fluids reported to date include thin-layer chromatography (Chen *et al.*, 1979, Chen *et al.*, 1982, Sanderson *et al.*, 1983, Kubota *et al.*, 1995), Gas chromatography with a flame ionization detector (Jones, 1994) and gas chromatography-mass spectrometry (Wolrath, 2001). Here we describe an enzymatic assay that:

- 1) can detect DA concentrations as low as 4 μM ;
- 2) exhibits a linear dose-response relationship over a wide range of DA concentrations;
- 3) can be used to test human vaginal samples;
- 4) is simple enough to be easily adapted to routine testing in the clinical laboratory.

Implementation of this assay on large numbers of vaginal samples from symptomatic and asymptomatic women will allow DA to be investigated as biomarkers of vaginal health.

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