Medical Microbiology and Immunology
https://doi.org/10.1007/s00430-019-00622-9

ORIGINAL INVESTIGATION

Rapid differential diagnosis of vaginal infections using gold nanoparticles coated with specific antibodies

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Received: 25 November 2018 / Accepted: 25 May 2019

Abstract

Vaginal infections caused by bacteria, Candida and *Trichomonas vaginalis*, affect millions of women annually worldwide. Symptoms and signs have limited value in differential diagnosis of three causes of vaginitis. Current laboratory methods for differential diagnosis are either expensive or time consuming. Therefore, in this work, development of a method based on gold nanoparticles has been investigated for rapid diagnosis of vaginal infections. Specific antibodies against three main causes of vaginal infections were raised in rabbits. The antibodies were then purified and conjugated to gold nanoparticles and used in an agglutination test for detection of vaginal infections. Finally, sensitivity and specificity of this test for diagnosis of vaginal infections were estimated using culture method as gold standard. Purification of antibodies from sera was confirmed by electrophoresis. Construction of nanoparticles was proved by TEM and FT-IR methods. Conjugation of antibodies to gold nanoparticles was confirmed using XPS method. Sensitivity and specificity of gold nanoparticles for diagnosis of Candida species were 100%, for Gardnerella were 100% and 93%, and for *T. vaginalis* was 53.3% and 100%, respectively. Gold nanoparticle-based method is a simple, rapid, accurate, and cost-effective test for differential laboratory diagnosis of vaginal infections.

Keywords Gold nanoparticles · Antibody conjugated · Vaginal infection · Diagnosis

Introduction

Vaginal infections affect millions of women annually worldwide [1]. The most common causes of vaginitis are bacterial vaginosis, vulvovaginal candidiasis, and trichomoniasis. Dysbacteriosis is implicated in 40–50%, vulvovaginal candidiasis in 20–25%, and trichomoniasis in 15–20% of cases [2]. Discharge is one of the most frequent gynecologic complaints. In the United States, vaginal infection account for more than 7% of patient visits to gynecologists' clinics [3, 4]. These infections are associated with several adverse health outcomes, such as preterm birth or delivery of a low-birth weight infant [1, 5, 6].

Dysbacteriosis (bacterial vaginosis) is a common condition, affecting many women annually [7, 8], and is associated with numerous health problems including preterm labor resulting in low-birth weight [9], pelvic inflammatory disease [10], and acquisition of the human immunodeficiency virus [11]. Malodorous vaginal discharge may be the only symptom of bacterial vaginosis, and many affected women

Edited by Jordi Vila.

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Published online: 10 June 2019

are asymptomatic [12]. In women with bacterial vaginitis, the concentration of anaerobic, *Gardnerella vaginalis* [13] and *Mycoplasma hominis* [14], is 100–1000 times higher than in normal women. However, these species are also found in subjects who do not have bacterial vaginosis and thus are not specific markers for disease [1].

Numerous studies have shown an association between bacterial vaginitis and adverse sequalae [15]. A meta-analysis investigation confirmed that bacterial vaginitis in pregnancy was associated consistently and significantly with an increased risk of prematurity [14].

Trichomonas vaginalis infection is a common sexually transmitted protozoal infection, with an estimated 180 million prevalent cases worldwide [16]. The diagnosis of *T. vaginalis* infection in women is often made by microscopic examination (wet mount) of a vaginal fluid specimen or by an incidental finding on a Papanicolaou test report. The sensitivity for these tests may be as low as 50% [17]. Other diagnostic options include culture and a DNA probe test, both of which have moderately higher sensitivity [18]. However, these options are not used routinely and are not cost effective for many clinicians.

Candida vaginitis is one of the most frequent infections of the female genital tract with a high incidence. Approximately 75% of sexually active women suffer at least one episode of Candida vaginitis and 10% of them have recurrent episodes [19]. Positive vaginal cultures for Candida species can be found in almost 15% of non-pregnant and 30% of pregnant women [20]. Among women with acute vulvovaginal candidiasis, *Candida albicans* accounts for 80–90% of the isolated fungal species, whereas other species are less frequent [21].

Patients with vaginal infection frequently continue to have symptoms following treatment. The main cause of this treatment failure is diagnostic errors. Many attempts have been made so far to determine the relationship between clinical criteria (symptoms and signs) and three causes of vaginitis (*T. vaginalis*, Candida species, and *G. vaginalis*). However, it is difficult to prove the etiology of vaginitis according to clinical criteria. It has been shown that symptoms are not differed among the three infections, and lack of vaginal odor in yeast infection is the only significantly different physical sign [20]. Therefore, it can be concluded that presenting symptoms and signs in vaginitis evaluation have limited value for differential diagnosis of vaginal infections [22].

In differential diagnosis of vaginal infections, laboratory diagnostic of vaginal infection is necessary for appropriate treatment and follow-up [23]. The laboratory diagnosis of vaginal infection in women is often made by microscopic examination (wet mount) of a vaginal fluid specimen. However, the sensitivity for these tests may be as low as 50% [24]. Other options for differential diagnosis of vaginal infections are culture and a DNA probe tests. These tests are more sensitive than wet smear method [25]. However, they are not used routinely, because they are time-consuming methods and they are not cost effective. To develop a rapid, sensitive and cost-effective method for simultaneous diagnosis of vaginal infections, in this work, differential diagnosis of these infections by gold nanoparticles coated with specific antibodies has been investigated.

Materials and methods

In this work, vaginal swabs were collected from 635 women with vaginal symptoms referred to gynecology clinics in Chaharmahal va Bakhtiari province of Iran in 2017. In the first step of this research, an agglutination test using gold nanoparticles coated with specific antibodies was developed for differential diagnosis of vaginal infections and in the second step sensitivity and specificity of the test was estimated.

Development of a gold nanoparticle-based method for differential diagnosis of vaginal infections

Antigen preparation

With informed consent, vaginal samples were collected from women referred to gynecology clinics. The samples were then cultured in appropriate mediums for each microorganism. Colombia agar medium, Sabra dextrose agar, and TYSI33 mediums were used for culturing *G. vaginalis*, *Candida* spp., and *T. vaginalis,* respectively. After growth in culture medium and performance of appropriate tests to confirm the infections, the above three agents were transferred to three individual tubes containing PBS. The microorganisms were then given three washes with PBS to remove constituent of the culture media. After that, the organisms were sonicated to prepare crude antigens. In case of *Candida* spp., species determination was not considered.

Preparation of specific antibodies

The prepared crude antigens along with adjuvant were injected to individual rabbits to raise poly-specific antisera. Complete Freunds' adjuvant was used for the first injection and incomplete one for the boosters. Following the third booster, a blood sample was prepared from all rabbits and presence of specific antibodies against causing agents of vaginal infection was checked using ELISA method, as we published before [26]. With the presence of appropriate level of specific antibodies, the rabbits were bled out and their sera was kept at − 20 °C until used. Antibodies in the sera were purified using salting out method. Antibody purification was then confirmed by electrophoresis method (sebia Capillarys 2 Flexiercing-France).

Preparation of gold nanoparticles and conjugation with specific antibodies

Gold nanoparticles with pendent carboxylic and alcohol functional groups were prepared [27] and then conjugated to specific antibodies against Candida species, *T. vaginalis* or *Gardnerella* species [28]. Briefly, the gold nanoparticles were synthesized using Laaksonen et al.'s method [27] with slight modification. To 200 mL of ethanol solution containing 2.7 mmol 0.3 mmol 16-mercaptohexadecanoic acid, 6 mL of a solution containing $HAuCl_4 \cdot 3H_2O$ (410 mg) in water (6 mL) was added. The solution was then cooled to 0 °C, and then, 20 mL of a freshly prepared aqueous solution containing 380 mg of NaBH4 was added. The resulting solution containing capped Au nanoparticles with pendant carboxylic acid functional groups was stirred for 3 h, and then, the material was allowed to precipitate to the bottom of the flask. The particles were then washed and dried under vacuum for 10 h and were examined using transmission electron microscopy (TEM) (Philips CM30, The Netherlands) and the FT-IR spectrum was obtained on a FT-IR spectrometer (6300-Jasco-Japan). The prepared gold nanoparticles functionalized with 16-mercaptohexadecanoic acid and were conjugated to the antibodies according to the method reported by Zhao et al. [29]. For this purpose, 100 mg of Au nanoparticles with pendant carboxylic acid functional groups, and 2 mg of antibody was added to a solution containing 0.25 g (1.3 mmol) 1-ethyl-3-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.25 g (2.2 mmol) *N*-hydroxysuccinimide in 5 mL 0.01 PBS (pH 7.4), and stirred at room temperature for 0.5 h. Material was then washed with 0.01 PBS. The obtained material was then characterized using μX-ray photoelectron spectroscopy (XPS) (PHI-5400, Physical Electronics USA).

Reaction of gold nanoparticles conjugated antibodies with the related antigens

On an agglutination slide, one drop (50 μL) of gold nanoparticles conjugated with anti-Candida antibodies was mixed with one drop (50 μL) of PBS containing 1×10^6 Candida cells/mL. With occurrence of agglutination, the test considered positive. This test was also repeated for *T. vaginalis* and *G. vaginalis*.

Determination of sensitivity and specificity of gold nanoparticles' method

Study population was women with signs and symptoms of vaginal infections referred to gynecology clinics in Chaharmahal va Bakhtiari province of Iran in 2017. With informed consent from each patient, five vaginal swabs were taken and used as follows.

The first swab was used for microscopic examination (wet smear). The second, third, and fourth swabs were used for specific culture medium for *T. vaginalis*, *Candida* spp., or *G. vaginalis,* respectively. The fifth swab kept at − 20 to be tested with gold nanoparticles' method. Sampling was continued until at least 30 positive samples of *T. vaginalis*, *Candida* spp. or *G. vaginalis* were collected. After that the developed gold nanoparticles method was used to examine the fifth swab. For this purpose, the swabs were shacked in 500 μL normal saline, then three drops (50 μL) of it put on agglutination slides on different spots. A drop (50 μL) of nanoparticles coated with anti *T. vaginalis*, anti-*Candida* spp. or anti *G. vaginalis* was added to drops 1–3, respectively, and shake for several seconds. With the formation of agglutination, the test considered to be positive. Finally, the results of nanoparticles' method were compared with the results of culture methods (gold standard) to estimate sensitivity and specificity of the newly developed nanoparticles test.

Analytical sensitivity and specificity of the gold nanoparticles method in detection of *Candida* spp. and *G. vaginalis*

For sensitivity, 0.5 McFarland standards were made for *Candida* spp. and *G. vaginalis* and different dilutions of the them were prepared and each dilution was then tested by gold nanoparticles method. For specificity gold nanoparticles, method was used for detection of *Escherichia coli*, *Klebsiella*, *Enterobacter aerogenes*, *Rodotrolla* spp., and *Geotrichum* sp.

Results

Preparation of gold nanoparticles

Gold nanoparticle was made and tested using transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR) (Figs. 1, 2).

Conjugation of gold nanoparticles with specific antibodies against *Trichomonas vaginalis*, *Candida* spp., or *Gardnerella vaginalis*

Three antisera raised against *T. vaginalis*, *Candida* spp., and *G. vaginalis* subjected to salting out method to purify the antibodies. As shown in Fig. 3, antibodies were partially purified. In the next step, gold nanoparticles were conjugated with the three specific antibodies. Conjugated of the gold nanoparticles with each specific antibody was confirmed using X-ray photoelectron spectroscopy (XPS) method (Fig. 4).

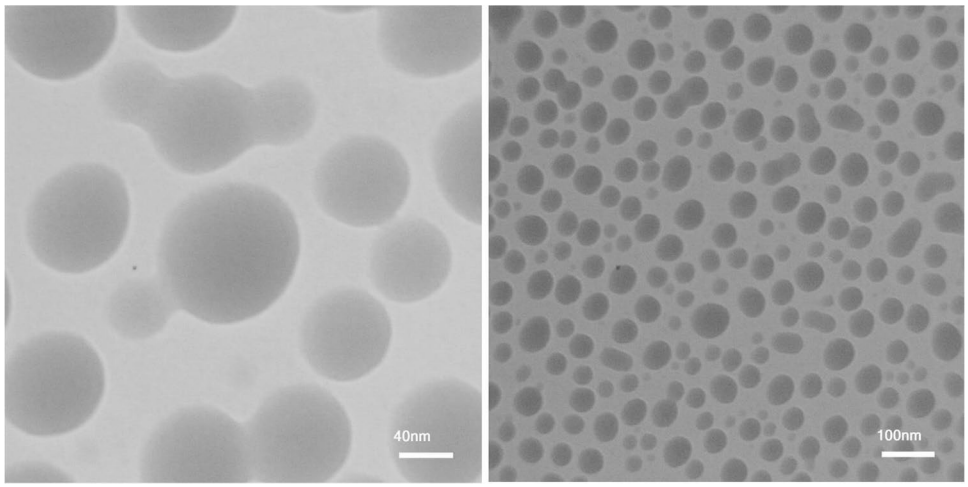


Fig. 1 TEM of prepared gold nanoparticles

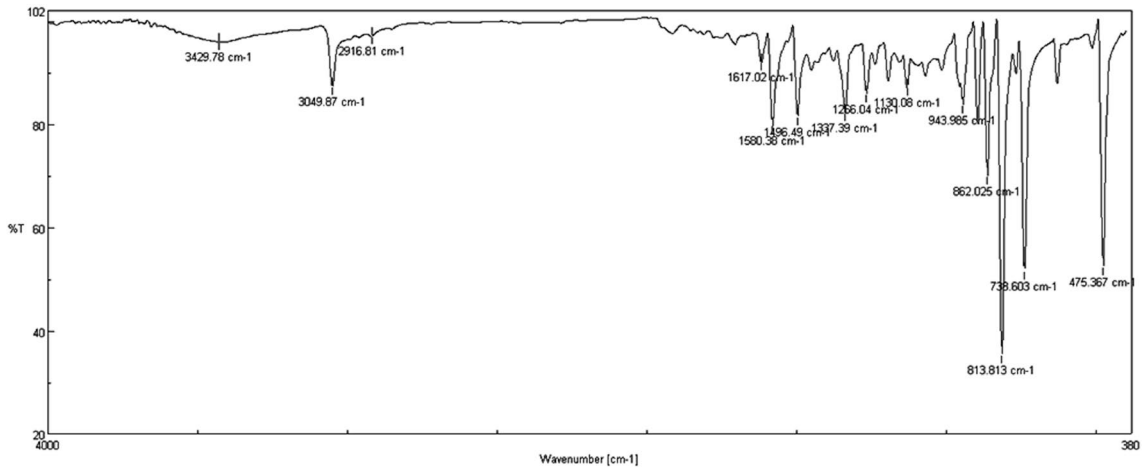


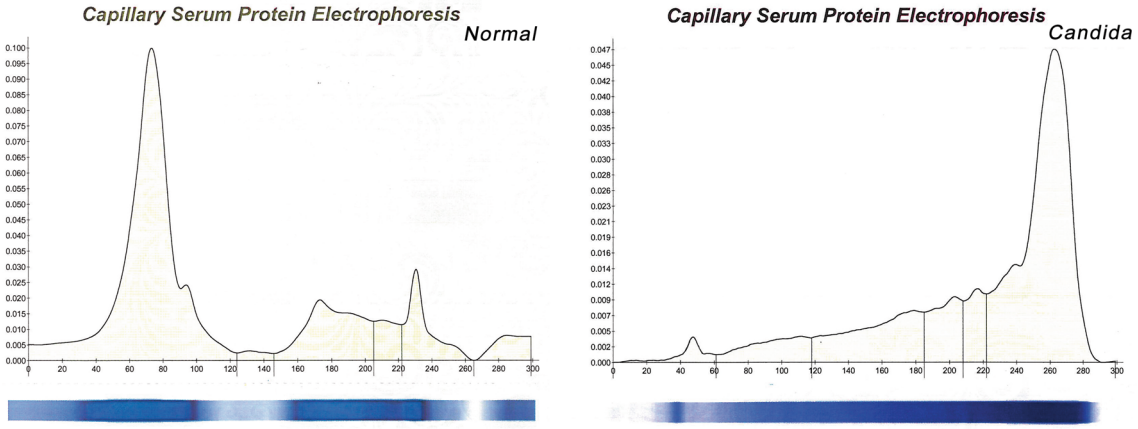
Fig. 2 FT-IR spectrum of functionalized gold nanoparticles with carboxylic acid groups. Peaks at various ranges related to different stretching and bending modes of the functional groups. Peaks at 3049.87 cm^{-1} are ascribed to C–H stretching of the alkyl groups and the peak at 1496.49 to the C–H deformation of the alkyl group. The peak at 1580.38 cm^{-1} is related to stretching of the carboxylic group. The peak at 1266.04 cm^{-1} corresponding to O–H bending was observed. A peak at 813.813 cm^{-1} is also present. The peak at 1130.08 cm^{-1} is characteristic of the out-of-plane O–H-bending mode and the peak at 3429.78 cm^{-1} is due to O–H stretching

Reaction of gold nanoparticles conjugated antibodies with vaginal infections agents

One drop (50 µL) of gold nanoparticles conjugated with anti-Candida antibodies was mixed with one drop (50 µL) of PBS containing Candida. As control, one drop (50 µL) of PBS alone was mixed with conjugated nanoparticles. In addition, one drop (50 µL) of vaginal samples was mixed with the conjugated nanoparticles. As shown in Fig. 5, after few seconds, a strong agglutination was observed. This test was also repeated for *T. vaginalis* and *G. vaginalis*.

Examination of the vaginal samples using wet smear, culture, and antibody-conjugated gold nanoparticles

635 vaginal samples were examined using wet smear and culture methods to detect 30 positive samples for *T. vaginalis*, *Candida* spp., and *G. vaginalis*, and then, the developed nanoparticles' method was performed on 90 positive samples (30 for each vaginal agent) and 90 negative samples. The results of this examination are summarized in Table 1.



Fractions	%		Ref. %	g/dl	Ref. g/dl
Albumin	**65.3**		55.8 - 66.1	3.0	4.0 - 4.8
Alpha 1	**1.3**	L	2.9 - 4.9	0.1	0.2 - 0.4
Alpha 2	**15.8**	H	7.1 - 11.8	0.7	0.5 - 0.9
Beta 1	**4.5**	L	4.7 - 7.2	0.2	0.3 - 0.5
Beta 2	**8.8**	H	3.2 - 6.5	0.4	0.2 - 0.5
Gamma	**4.3**	L	11.1 - 18.8	0.2	0.8 - 1.4

Fractions	%		Ref. %	g/dl	Ref. g/dl
Albumin	**2.4**	L	55.8 - 66.1	1.5	4.0 - 4.8
Alpha 1	**7.0**	H	2.9 - 4.9	4.4	0.2 - 0.4
Alpha 2	**15.7**	H	7.1 - 11.8	9.8	0.5 - 0.9
Beta 1	**8.4**	H	4.7 - 7.2	5.3	0.3 - 0.5
Beta 2	**6.1**		3.2 - 6.5	3.8	0.2 - 0.5
Gamma	**60.4**	H	11.1 - 18.8	37.8	0.8 - 1.4

Fig. 3 Electrophoresis of purified antibodies against Candida in comparison with electrophoresis of the normal sera

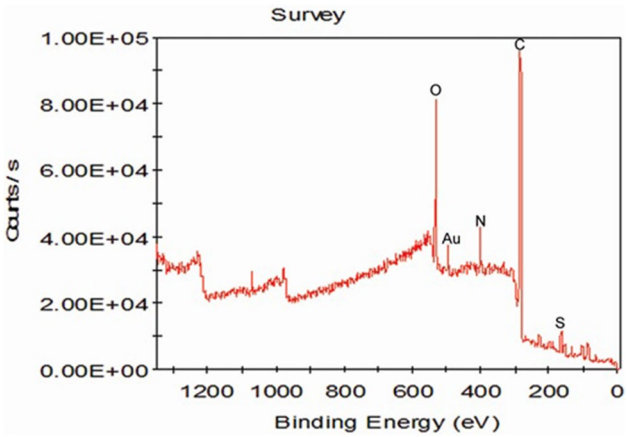


Fig. 4 X-ray photoelectron spectrum of gold nanoparticle with the attached antibody anti-*Candida* spp. This figure indicates the presence of the antibody on the nanoparticles by X-ray photoelectron spectroscopy (XPS). A peak at ~ 400, related to nitrogen present in the sample from antibody, is observed

Fig. 5 Agglutination between Candida (1), PBS (2), and a vaginal sample (3) and the gold nanoparticles conjugated with anti-Candida

Sensitivity and specificity of gold nanoparticle method for diagnosis of vaginal infections

Sensitivity and specificity of gold nanoparticle method for detection of *T. vaginalis*, *Candida* spp., and *G. vaginalis* in vaginal samples were estimated. Gold nanoparticles' method has a high level of both sensitivity and specificity for detection of *Candida* spp. and *G. vaginalis*. However, the sensitivity of the test for detection of *T. vaginalis* was 53.33%. More details about sensitivity and specificity of gold nanoparticles method are shown in Table 2.

Results of analytical sensitivity and specificity of the gold nanoparticles method in detection of *Candida* spp. and *Gardnerella vaginalis*

Gold nanoparticles' method was able to detect as much as 25 Candida cells in 1 ml (0.25×10^2 CFU/mL). The analytical sensitivity of the test for *G. vaginalis* was 1×10^2 CFU/mL. Therefore, low inoculum of the test for Candida and *G. vaginalis* was 0.25×10^2 CFU/mL and 1×10^2 CFU/mL, respectively. In analytical specificity, the gold nanoparticles' method did not detect *Escherichia coli*, *Klebsiella*, *Enterobacter aerogenes*, *Rodotrolla* spp., and *Geotrichum* sp.

Table 1 Comparison of results of wet smear, culture and Gold nanoparticles method for detection of *Trichomonas vaginalis*, Candida, and *Gardnerella vaginalis*

	Gardnerella vaginalis		*Candida* spp.		*Trichomonas vaginalis*	
	Positive	Negative	Positive	Negative	Positive	Negative
Nano method						
Positive	30	2	30	0	16	0
Negative	0	28	0	30	14	30
Total	30	30	30	30	30	30

Table 2 Sensitivity and specificity of gold nano method in detection of vaginal infections

	Sensitivity %	Specificity %	Positive predictive value %	Negative predictive value %
Gardnerella vaginalis	100	93.3	93.75	100
Candida spp.	100	100	100	100
Trichomonas vaginalis	53.33	100	100	36.3

Discussion

To provide a rapid and accurate test for differential diagnosis of vaginal infections in human, in this work, a gold nanoparticle method was developed. The test was rapid, and after only few seconds, a strong agglutination is developing between antigen and the conjugated antibodies. Results of this investigation showed that gold nanoparticle method has a high level of sensitivity and specificity for detection of human vaginal infections caused by *Candida* spp. and *G. vaginalis*.

Itching was the only symptom more frequently noted among symptomatic patients [20]. In an investigation, it has been shown that the clinical diagnosis of vaginal infection is inadequate in diagnosis of causative agents of vaginal infections and should be confirmed with an appropriate laboratory test [30]. Precision and accuracy of microscopy of vaginal fluid method are poor [31] and misdiagnosis creates stress for the patient, delays appropriate intervention, and places a financial burden on the health care system. In an investigation, a nucleic-acid probe-based test was developed for differential diagnosis of vaginal infections. Sensitivity and specificity of more than 90% were reported for this test in detection of *G. vaginalis*, *Candida* spp., and *T. vaginalis* in vaginal specimens [32]. Although this test is both sensitive and specific, but it is an expensive method and needs special equipment to do the test. In another investigation, the sensitivity of clinician microscopy for diagnosis of vulvovaginal candidiasis, vaginal trichomoniasis, and bacterial vaginosis was 39.6%, 90.4%, and 75.0%, respectively, while the sensitivity of a DNA probe method was 75.0%, 95.7%, and 86.5%. The specificity of conventional and DNA probe method was 96.6%, 76.5%, and 70.8%, and 98.5%, 95.4%, and 60.7%, respectively [33]. Daniel et al. showed that the sensitivity of wet mount diagnosis of trichomoniasis was 62%, and of Candida by microscopy was 22% [13]. They also concluded that symptoms alone should not be used to direct treatment of vaginal infection [13].

In another work, it has been shown that molecular detection of *G. vaginalis* has similar sensitivity and specificity to Gram stain method [34]. Performance of molecular methods is time consuming and needs especial equipment.

Culture methods are used for diagnosis of *G. vaginalis*, Candida species, and *T. vaginalis*. This method is time consuming, and do not provide timely results. A DNA probe analysis of vaginal fluid for *G. vaginalis*, Candida species, and *T. vaginalis* is sensitive and specific for detection of these agents [35]. However, DNA probe analysis is an expensive method and needs especial equipment.

Molecular methods have been used as complements to conventional methods and providing more accurate results in less time (1.5–3 h). Therefore, they are considered as accurate and quick tests for diagnosis of candidiasis. However, these tests are expensive and lack appropriate standardization [36]. Trama et al. developed a PCR-based method for diagnosis of Candida species in vaginal samples. They showed that this test provided high level of both sensitivity and specificity for diagnosis of vaginal candidiasis [37].

Sensitivity and specificity of 100% have been reported for PCR method in detection of *T. vaginalis* in vaginal samples [38]. In another work, it has been shown that vaginal swab ATV TMA was significantly more sensitive than wet mount or culture in detection of *T. vaginalis* in women, while in men urethral swab, ATV TMA was significantly more sensitive than culture or PCR [39]. In another work, Lisa et al. showed that the sensitivity and specificity of PCR using vaginal samples for detection of *T. vaginalis* were 89 and 97%, respectively [40]. It has been shown that PCR could

be method of choice for detection of *T. vaginalis* in vaginal samples [41]. In another work, it has been reported that real-time PCR assay is sensitive and specific for the detection of *T. vaginalis* DNA in vaginal samples [42]. In contrast lower sensitivity of PCR method for detection of *T. vaginalis* has been reported by Crucitti et al. [43].

Time, cost, and accuracy are important criteria of a laboratory test for detection of vaginal infections agents in clinical samples. In this investigation, we developed a quick, cost effective, and accurate test for laboratory differential diagnosis of vaginal infections simultaneously. Therefore, conventual and molecular methods which are either time consuming or expensive can be replaced by this test. Moreover, this method is easy to do with no need to any equipment and can also be performed in gynecological clinics.

Conclusion

Gold nanoparticle method developed in this work has a high level of specificity and sensitivity for detection of *Candida* and *Gardernela* spp. in vaginal samples. Simultaneous differential diagnosis of vaginal infections simply and quickly is the most important advantages of this test.

Acknowledgements This work supported by Grant from Isfahan University of Medical Sciences (Grant no. 563284).

Compliance with ethical standards

Conflict of interest None of the authors has conflict of interests.

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