

RESEARCH ARTICLE

Diagnostic Value of the Care™ HPV Test in Screening for Cervical Intraepithelial Neoplasia Grade 2 or Worse

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Abstract

Background: Cervical cancer is the fourth leading cause of cancer death in women worldwide. Persistent infection with a high risk human papillomavirus (HR-HPV) is the main etiological factor, so that early early detection of HR-HPV is very important. The aim of this study was to investigate the efficacy of Care™ HPV, a new method, as compared with Pap smear, PCR, and biopsy for screening purposes. **Material and Method:** In this cross-sectional study, 200 sexually active women aging from 25-50 years referred to the oncology clinic of Shahid Sodoughi Yazd Hospital in 2015 with a variety of cervix epithelial lesions or a need for colposcopy were enrolled. Results for Care™ HPV test (cervical), Pap smear, PCR, and biopsy were analyzed using SPSS 15 software and chi-square test, McNemar, and ROC curve analysis. Qualitative variables were compared using a Chi-square test. **Results:** Care™ HPV test sensitivity in detecting cervical intraepithelial neoplasia grade II (CIN-II) and also positive and negative predictive values were higher as compared to with other tests ($p < 0.05$). The Pap smear test specificity was highest. There was no significant differences between Care™ HPV and PCR tests regarding detection of HPV-DNA in cases of CIN-II and worse ($p > 0.05$). **Conclusion:** The Care™ HPV test has high sensitivity and predictive values for detecting HPV infection, with higher efficacy than the Pap smear test for tracking CIN-II. Therefore it may be recommended for use as a screening test in low-income areas.

Keywords: Care™ HPV- human papillomavirus- cervical cancer- CIN-II

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Introduction

Cervical cancer is a global health problem (Munoz et al., 2006). According to global reports, cervical cancer is the fourth leading cause of cancers death in women worldwide and the fourth most common cancer in terms of incidence among women around the world (Torre et al., 2015). The prevalence of this cancer is about 470,000 new cases and about 233,000 deaths among women around the world. According to global statistics, nearly 500,000 people around the world have been diagnosed with this disease, which about 80 % are in developing countries and almost half of them lose their lives (Stewart et al., 2015).

In developed and industrial countries due to screening programs and proper infrastructure strategies, incidences of this cancer are remarkably on a decline (Torre et al., 2015). On the other hand, in countries with low-income and in developing countries, the incidence of this cancer and associated pre-cancers are on a rise for various reasons including lack of proper quality control systems, continuous screening infrastructure, and low efficiency monitoring systems with poor tools used during performance (Wentzensen et al., 2015).

This cancer is considered one of the most important diseases caused by the human Papillomavirus (HPV).

HPV, especially high-risk types (high-risk HPV), is a cause factor for this cancer and for related lesions such as Cervical Intraepithelial Neoplasia (CIN) and Atypical squamous cells of undetermined significance (ASCUS) (Huh et al., 2015; Giorgi-Rossi et al., 2015). The incidence of this virus is substantially growing (Ahdieh et al., 2001).

Screening programs and routine tests such as Pap smear and Tien Prep have false negative results and require skilled people for diagnoses (Lorenzi et al., 2013) for example, previous studies have shown that the sensitivity of cytopathology Pap smears to detect cancer or pre-cancerous is equal to 53% (Cuzick et al., 2006), which leads up to a 50% increase in false negative results (Sprenger et al., 1996), and with 80% sensitivity and 98% specificity (Soost et al., 1991).

Cervix persistent infection is caused by approximately 13 virus types (Sundström et al., 2015). Screening programs including the HPV- DNA tests were approved by FDA (Kang et al., 2014) to decrease the incidence of cervical cancer in 4-5 recent years. In addition, over the last 8 years the mortality rate has decreased (Torre et al., 2015; Patra et al., 2015). DNA-HPV test is a useful tool in screening women with high risk factors and has more sensitivity than Pap smear cytology (Cox and Cuzick, 2006; Franco and Cuzick, 2008; Gravitt et al., 2010;

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Shastri et al., 2005; Basu et al., 2013; Zhao et al., 2012), among these methods it can point to Care™ HPV, which has FDA confirmation with confirmed sensitivity and specificity (Lorenzi et al., 2013; Qiao et al., 2008; Zhao et al., 2013; Gage et al., 2012; Trope et al., 2013).

Care™ HPV is a quick, efficient and a simple method that does not require special laboratory equipment (Ying et al., 2014). It doesn't depend on a person's skills, and in comparison to Pap smear test, it is more sensitive and operational (Zhao et al., 2012; Ho et al., 1998). It takes approximately about 2 hours and 30 minutes. The short time of this test allows doctors to track patients in a short visit and give more attention to them. The accuracy of this method is 90% and is very economical. In this method after pouring high risk types of HPV- DNA samples, antibodies specifically bind to it then are read via chemiluminescence signals (Ying et al., 2014). The DNA- HPV test improved CIN II and worse detection in developing countries, particularly in rural areas, in fact it preferred in rural areas to cytology test where facilities are not available (Torre et al., 2015; Lazcano et al., 2011). These results indicate that Care™ HPV could be more suitable for the primary screening of cervical cancer and associated pre-cancers. Therefore, the aim of this study was to compare results of cervical lesions checkings by Pap smear (liquid based), PCR, biopsy, and Care™ HPV.

Materials and Methods

Study design and sample size

This study was designed as a diagnostic cross-sectional study and was performed in Oncology Clinic of Shahid Sodoughi Yazd Hospital as a referral center in south of Iran during 2014-2015.

According to previous similar studies (Lorenzi et al., 2013; Qiao et al., 2008) and considering 95% confidence interval, 80% sensitivity and specificity, and 8% estimation error, a required sample size of 200 people was calculated. We used random sampling method and eligible clients were selected.

Inclusion criteria

Sexually active women, aging from 25-50 years with a variety of cervical epithelial lesions or colposcopy need cases were referred to the oncology clinic of Shahid Sodoughi Yazd Hospital.

Exclusion criteria

Women who undergo hysterectomy or had been diagnosed for cancer, or were precancerous, or had performed any type of cervical surgery. In addition, samples that were not collected and store well, as well as women with temporarily menstrual were excluded.

In this study, four methods including PCR, Pap smear, Biopsy, and Care™ HPV for screening and diagnosis of cervical cancer and associated lesions were compared. There was a huge focus on Care™ HPV, being considered as a new method. In the following the four methods are explained.

PCR method to identify genome of HPV

Digestion buffer and proteinase K preparation:

Per 100 µL of consumer digestive buffer, 2.5 µL Proteinase K was added.

Nucleic acid (DNA) purification

We used Amplisense extraction kit to extract viral genome.

Control of extracted DNA

To evaluate the physical condition of extracted DNA and all extraction steps, we used the human β-globin gene, and for papillomavirus DNA detection we used Amplisense company kit. We used distilled water injections and DNA extracted from HeLa cells respectively as negative and positive controls.

PCR reaction for investigate quality of extracted DNA

The reaction mixture composition in a final volume of 50 µL and the desired temperature cycle for each PCR sample to check PCR extraction quality with β-globin gene primers (Table A) are respectively listed in Table B and C:

PCR product electrophoresis was done in a 2% agarose gel containing ethidium bromide and was examined with Gel Doc device; that PCR product was 110bp size.

Final step

For HPV genome detection, we used PCR method by using the AmpliSens® PCR diagnostic kit. The Papilloma virus genome detection product was 450bp. Electrophoresis was performed in a manner that mentioned earlier and was evaluated with a device emitting ultraviolet radiation.

Pap Smear (liquid-based)

Before sampling the following issues should be observed:

1. Sampling doesn't occur during menstruation or when secretions are very high and if there was a clear infection
2. forty-eight hours before sampling, sex didn't take place.
3. Vaginal ointments wasn't used at least a week before sampling.

Sample preparation steps

In the beginning, 2-4 ml of cleaning solution was added to a sample and incubated 1 hour in room temperature. Then, the tube was centrifuged 20 min at 3000 rpm, then sediment removed and combined with 300-500 µL of cellular base solution. Then, 50 µL of the sample were put into two circles with a diameter of 1.5-2 cm spread on each slide, and finally dried for 1-2 hours at room temperature.

Staining method

Pap smear slides were stained by Papanicolaou staining liquid. A basket containing Pap smear liquid slides were placed into solutions with the order that is said bellow.

Ethanol 96° (20 minutes)→ rinse with water → hematoxyline color (5min)→ rinse with water→ acid-alcohol 1%(5-10 seconds)→ rinse with

water→alcohol-ethanol96 °(8min)→OG6 color(4min)→alcohol-ethanol96 °(8min)→EA50 color (5min)→absolute ethanol (6 min)→ drying at room temperature(10 min(→ alcohol xylol for transparent process→interpreted by a pathologist

Biopsy method

Cervical cancer diagnosis through biopsy involves removing a small piece of cervix tissue and then in vitro evaluation. In most cases, a biopsy is done with a local anesthetic in a doctor's office. In the most common method, a wooden or plastic spatula is stretched on a cervix aperture and then a soft brush is used in the lower part of uterus. The sampling time is short and usually with no pain. There may be some discomfort, but this feeling soon passes.

Principles of Care™ method

A nucleic acid hybridization assay is done in chemiluminescence plates as nucleic acid specific antibodies of 18 low and high risk HPV strains connected. Then with chemiluminescence signals tracing, qualitative detection of nucleic acids HPV strains takes place. Suspicious samples that contain HPV- DNA will hybrid with HPV-specific RNA probe. Then formed RNA/DNA hybrid binds to alkaline phosphatase conjugated antibodies when multiple alkaline phosphate molecules bind to these hybrids, signal amplification caused, which means that when substrate is broken, light is emitted. This light is proposed as a relative light unit (RLU) in luminator. Therefore, the intensity of emitted light interpreted by the presence or absence of HPV-DNA. RLU measurement equal or greater than the standard Cut-off shows the presence of HPV- DNA sequences in samples. RLU less than the Cut-off criteria indicates the absence or low levels of HPV-DNA or lower than test detection. RLU ratio to at least positive control RLU (RLU/CO) is used as a diagnostic tool.

Ethical considerations

All moral codes regarding the study and the confidentiality of information were observed, and also an informed consent form was obtained from the subjects. No additional processes was performed on the patient. This study was approved by the ethic committee Shahid Sodoughi Yazd University of Medical Sciences (ethical code: R.SSU.MEDICINE.REC.1394.148).

Statistical analysis

Collected data entered, and analyzed by SPSS version 15. Qualitative variables were evaluated using the Chi-Square test. Then HPV positive detection rate by PCR and Care™ were compared. However, the sensitivity, specificity, positive and negative predictive values were analyzed using the McNemar test. $P < 0.05$ was considered significant.

Results

In this study, 200 individuals participated and their demographic and disease data can be seen in Table 1.

Patient's age ranged from 25-50 years with 37 ± 7.5 years in average.

The quality of extracted DNA and HPV genome screening

To evaluate the physical condition of extracted DNA, the human β -globin gene was used in the extraction process. PCR bands of this gene is 110 bp. In Figure 1, the number of samples that have been randomly selected show a specific band β -globin gene. A few examples of the positive samples for HPV-DNA can be seen in Figure 2.

The results of PCR and Care™ HPV methods can be seen in Table 2. The PCR positive results in detection CIN-II and worse was more than Care™ results. But there was no significant difference between results of PCR and Care™ methods in CIN-II and worse detection ($p=0.39$).

In the following, we can see and compare the frequency of Care™ HPV test based on PC

R test in classified age groups in Table 3. There was no significant difference between PCR and Care™ HPV results in older and younger than 35 years old in detecting CIN-II and worse ($p > 0.05$).

The results of PCR and Pap smear test can be seen in Table 4. The PCR positive results in detection CIN-II and worse is more than Pap smear test. There was no significant difference between results of PCR and Pap

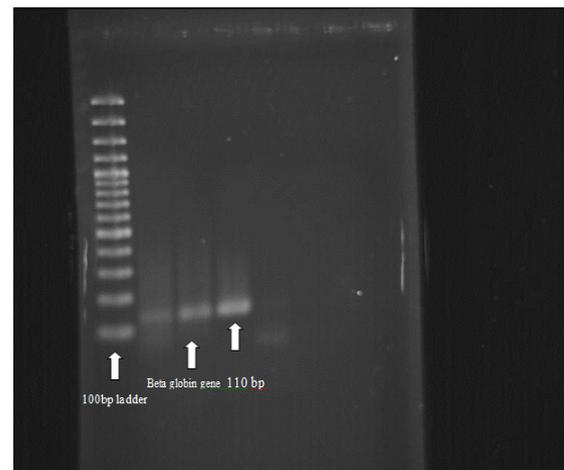


Figure 1. PCR Products of PC03/PC04 Primers, Respectively from the Left; 100bp Ladder, β -Globin Gene, Band 110bp

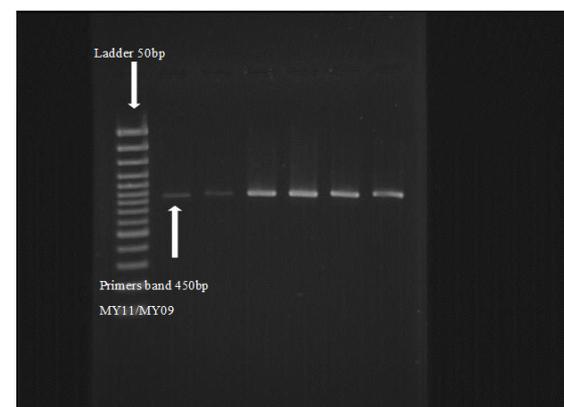


Figure 2. PCR Products of MY09/MY11 Primers, Respectively from the Left; 50bp Ladder, 450 Bp for Detection Virus Genome in Cervical Tissue Samples

Table1. Distribution of Demographic Data and the Results of Pathology and Pap Smear Tests

		Women		
Variable	Variable data	Percent (%)	Number	
Age	<35	45.5	91	
	>35	54.5	109	
Pop	AUSUS	49	98	
Smear	CIN I	2.5	5	
	Normal	21.5	43	
	HSIL	1.5	3	
	LSIL	14	28	
	CIN II	1	2	
	ASC-H	9.5	19	
	CIN III	1	2	
	CIN I	22	44	
	Biopsy	CIN II	7	14
		CIN III	2.5	5
Normal		17.5	35	
Metastatic carcinoma		0.5	1	
Insitu sec		0.5	1	
	Cervicitis with squamous metaplasia	37	71	
	Cervicitis koilocytic	13	29	

smear methods in CIN-II and worse detection (p=0.24).

We can see and compare Pap smear test results frequently according to PCR test in classified age groups in Table5. The PCR positive results in tracking CIN-II and worse is more than Pap smear test. There was a significant difference between the rate of PCR positive results and Pap smear in older and younger than 35 years old in detecting CIN-II and worse (p=0.00).

The results of Care™ HPV and Pap smear test can be seen in Table 6. The Care™ HPV positive results in detection CIN-II and worse is more than Pap smear test. There was no significant difference between the PCR and

Table 2. determine and compare Care™ test results frequently distribution according to PCR test in detecting cervical epithelial lesions CIN II and worse

	Variable	Results (%)		Total
		Positive	Care™ HPV Negative	
PCR Results (%)	Positive	33 (84.6)	6 (15.4)	39 (100)
	Negative	4 (2.5)	157 (97.5)	161 (100)
Sensitivity: 0.842		95% CI (0.917 – 0.999)		
Specificity: 0.958		95% CI (0.917 – 0.999)		
Positive predictive value: 0.846		95% CI (0.767 – 0.917)		
Negative predictive value: 0.941		95% CI (0.939 – 0.999)		

Table 3. Determine and Compare the Frequency of Care™ HPV Test based on PCR Test in Classified Age Groups

Age	PCR Test Results	Test Results		HPV (%) Total
		Positive	Care™ Negative	
<35	Positive	16 (84.2)	3 (15.8)	19 (100)
	Negative	3 (4.2)	69 (95.8)	72 (100)
>35	Positive	17 (85)	3 (15.0)	20 (100)
	Negative	5 (5.6)	84 (94.4)	89 (100)

Table 3 A. Sensitivity, Specificity, Positive and Negative Predictive Value and Negative in Younger than 35 Years Old

Sensitivity: 0.842	95% CI (0.767 – 0.917)
Specificity: 0.958	95% CI (0.917 – 0.999)
Positive predictive value: 0.842	95% CI (0.767 – 0.917)
Negative predictive value: 0.958	95% CI (0.917 – 0.999)

Table 3 B. Sensitivity, Specificity, Positive and Negative Predictive Value and Negative in Older than 35 Years Old

Sensitivity: 0.85	95% CI (0.783 – 0.917)
Specificity: 0.944	95% CI (0.901 – 0.987)
Positive predictive value: 0.773	95% CI (0.694 – 0.852)
Negative predictive value: 0.965	95% CI (0.931 – 0.999)

Pap smear results in detecting CIN-II and worse (p=0.64).

Based on the results and contingency coefficient KAPPA = 0.02 and P = 0. 003, we conclude that Care™ HPV and Pap smear test results have agreed in very low level.

We can see the comparison of sensitivity, specificity, and the predictive values of tests to detect CIN-II and worse by a variety of tests in Table 7. Care™ HPV sensitivity in detecting inter-epithelial neoplasia of grade II and worse is superior than other tests.

In addition, the specificity of the Pap smear test for this type of tracking is more than others. Care™ HPV positive and negative predictive value was higher than

Table 4. Determine and Compare Pap Smear Test Frequently Distribution Based on PCR Test in Detecting Cervical Epithelial Lesions CIN-II and Worse

	Variable	Pop Smear Results (%)		Total
		Positive	Negative	
PCR Results (%)	Positive	31 (20.5)	8 (79.5)	39 (100)
	Negative	121 (24.8)	40(75.2)	161(100)
Sensitivity: 0.794		95% CI (0.422 – 0.621)		
Specificity: 0.248		95% CI (0.422 – 0.978)		
Positive predictive value: 0.203		95% CI (0.422 – 0.978)		
Negative predictive value: 0.833		95% CI (0.422 – 0.978)		

Table 5. Determine and Compare Pap Smear Test Results Frequently Distribution According to PCR Test in Classified Age Groups

Age	PCR test results	Pop Smear Test	Results (%)	Total (%)
		Positive	Negative	
<35	Positive	24 (88.9)	3 (11.1)	27 (100)
	Negative	70 (76.9)	21 (23.1)	91 (100)
>35	Positive	15 (75.0)	5 (25.0)	20 (100)
	Negative	43 (69.3)	19 (30.7)	62 (100)
P value	P=0.00			

Table 5 A. Sensitivity, Specificity, Positive and Negative Predictive Value in Younger than 35 Years Old

Sensitivity: 0.889	95% CI (0.832 – 0.946)
Specificity: 0.231	95% CI (0.155 – 0.307)
Positive predictive value: 0.255	95% CI (0.176 – 0.334)
Negative predictive value: 0.808	95% CI (0.737 – 0.879)

Table 5 B. Sensitivity, Specificity, Positive and Negative Predictive Value in Older than 35 Years Old

Sensitivity: 0.75	95% CI (0.656 – 0.844)
Specificity: 0.306	95% CI (0.206 – 0.406)
Positive predictive value: 0.258	95% CI (0.164 – 0.354)
Negative predictive value: 0.792	95% CI (0.704 – 0.88)

Table 6. Determine the Frequency of Care™ HPV and Pap Smear Test Results in Detection CIN-II and Worse

Variable	Pop Smear	Results (%)	Total
	Positive	Negative	
Care™ HPV (%)	Positive	27 (80)	38 (100)
	Negative	123 (74.4)	162 (100)

other tests ($p < 0.05$).

Discussion

HPV infection is one of the main causes of cancer deaths that has resulted from persistent infections with HPV carcinogenic genotypes (Cox and Cuzick, 2006). Therefore, detecting high-risk genotypes with molecular testings in cervical samples is a type of cervical cancer prevention (Zhao et al., 2011; Qiao et al., 2008; Longatto-Filho and Schmitt, 2007). Nowadays, molecular basis tests are more developed for screening women at risk and for HPV detection. Due to high sensitivity and reproducibility, HPV-DNA tests in comparison to cytology tests have been proven in tracking people with CIN-II (Qiao et al., 2008).

In this study, the detectability of HPV-DNA in studied women was above 15% (17.5%); this rate compared to the PCR method did not show any significant differences and the HPV-DNA detection rate in the PCR test is found to be nearly the same (19.5%). The results show that the PCR method and Care™ HPV are nearly equivalent.

Care™ HPV test sensitivity and specificity to detect inter-epithelial neoplasia CIN II and worse, compared to

other tests especially Pap smear is higher. In addition, positive and negative predictive values in Care™ HPV were higher than others. As in this study, the criteria for negative predictive value in patients with CIN-II and worse was about 96.2%, therefore, this method of screening is somewhat a 5-year guarantee. The test is designed for low-income areas and women over 35 years. In this study, there was no significant difference found between the Care™ HPV and PCR positive trace rate. And possibly the differences in sensitivity of Care™ HPV in this study with other studies are the small sample sizes and the lack of patients with CIN-II and worse grades. However, the Care™ HPV method can be used as an accurate measure as due to the cost-effectiveness, little time to perform, and the availability for pre-cancer screening in developing countries.

This method can trace the 14 important viral genotypes in a limited time. As being said, it is very convenient to search for pre-cancers in women at risk. The results of the study showed over 90% positive and negative predictive values and high sensitivity compared with other tests to detect CIN-II and worse grades. These results are consistent with the results of Qiao et al., (2008) in China, which showed over 90% sensitivity and higher specificity for Care™ HPV than liquid-based cytology in tracing CIN-II and worse grades. In this study, only a woman with HSIL cytology was negative for HPV-DNA. On the other hand, approximately 75% of patients with LSIL were without HPV-DNA, which it shows that all CIN high grades are not associated with high-risk HPV. This result is consistent with a study conducted by Lorenzi et al., (2013) in Brazil.

This study showed that Care™ HPV sensitivity is greater than PCR for low-risk and high-risk types of HPV detection especially in patients older than 35 years. On the other hand, this tests sensitivity in women older

Table 7. Compare the Sensitivity, Specificity and Predictive Value of Tests for Detection CIN-II and Worse

Type of test	sensitivity	Specificity	Positive predictive value	Negative predictive value
Pop Smear	24.8	87.3	20.3	83.3
Care™ HPV	97.9	78	88.5	96.2

than 35 years compared to the women that are less than 35 years old is more than Pap smear test. These results are similar to Lorenzi et al. study's outcome in Brazil (Lorenzi et al., 2013).

However, the sensitivity of the test depends on vaginal or cervical sampling. Labani et al. study in India presented that the sensitivity of Care™ HPV cervical samples used to detect CIN-II and worse grades is more than vaginal samples. On the other hand, this study is based on the tracing of HPV in cervical samples, so this may affect the sensitivity compared to vaginal samples (Labani et al., 2014). However, sampling method can also have an effect on the results. As shown in a study, sensitivity differs in self-collected versus clinician-collected samples. But all samples in our study were clinician-collected samples and it would be better 2 type sampling method were examined.

In Lorenzi et al. study Care™, HPV sensitivity was shown over 90%, which is consistent with this research's results (Lorenzi et al., 2013). There is possibility that some people in this study had no HPV related diseases; on the other hand, we can say that it is possible for virus copy rates in negative samples to be less than the Care™ HPV trace limitation that requires lower RLU.

However, in this study it was observed that two women weren't tested with the Pap smear in the previous year, had cancer this year. One of the women has metastatic carcinoma, but the other patient has Insitu scc with positive Care™ HPV.

After genotyping the positive sample, the presence of HPV type 16 was approved. Given that last year this patient had no sign of cancer, but this year they have cancer, means that if they had been tested with a sensitive test like Care™ HPV then it would've been a proper prevention stage to prevent cancer.

Suggestions

1. Perform Care™ HPV test by investigating the sensitivity and specificity in larger sample sizes.
2. Compare self and clinical sampling with Care™ HPV test results to trace CIN-II and worse grade
3. Perform Care™ HPV test as a routine screening test for women over 35 years.

Due to high sensitivity, economical and comfortable of Care™ HPV test and on the other hand, high positive and negative predictive value of this test, so it is better in the future years, used in women in developing countries where are not the idyllic health conditions as a screening tests for prognosis and diagnosis CIN-II and worse.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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Authors' contributions

All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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