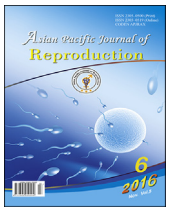
Contents lists available at ScienceDirect

Asian Pacific Journal of Reproduction

journal homepage: www.apjr.net



Original research http://dx.doi.org/10.1016/j.apjr.2016.10.007

Microorganism spectrum of nonspecific vaginitis in women of infertile couples recognized by s-IgA uterine cervix secretion

Muhammad Anas[1*,#], I Wayan Arsana Wiyasa[2,#], Slamet Riyanto[3], Teguh Wahyu Sardjono[4,#], Aulani'am Aulani'am[5,#], Sumarno Reto Prawiro[3,#]

[1]*Doctorate Program of Biomedicine, Faculty of Medicine, Brawijaya University, Malang, Indonesia*

[2]*Laboratorium of Obstetrics and Gynecology, Saiful Anwar Public Hospital, Malang, Indonesia*

[3]*Laboratorium of Microbiology, Faculty of Medicine, Brawijaya University, Malang, Indonesia*

[4]*Department of Parasitology, Faculty of Medicine, Brawijaya University, Malang, Indonesia*

[5]*Laboratorium of Biology, Faculty of Veterinary, Brawijaya University, Malang, Indonesia*

ARTICLE INFO

Article history:
Received 12 Aug 2016
Received in revised form 24 Oct 2016
Accepted 24 Oct 2016
Available online 3 Nov 2016

Keywords:
Antibiotic sensitivity test
Bacteria
Cross-immunity
Infertile women
Nonspecific vaginitis

ABSTRACT

Objective: To explore the possible causes of infertility that occurs in Mojokerto, East Java, Indonesia.

Methods: The study was conducted by collecting biographical data in general and through clinical examination of all infertile couples. Other collected data included profiles of bacteria found in the vagina and an antibiotic sensitivity test of bacteria that can be isolated. To determine the possibility of antibodies against sperm a western blot examination of bacterial proteins in fluid secretions obtained from the vagina was carried out first.

Results: The results show that microorganisms were identified from the female reproductive tract of infertile couples dominated by *Staphylococcus aureus* (*S. aureus*) (27%) and *Escherichia coli* (27%), and sensitivity tests of antibiotics vary greatly. One of the microorganisms particularly *S. aureus* that found in large number was done western blotting using s-IgA cervix uteri as an antibody recognized Omp's with molecular weight, 52 kDa, and 49 kDa.

Conclusion: One of the bacteria species *S. aureus* that infects the vagina and causes nonspecific vaginitis can provoke an adaptive immune response by producing s-IgA. Difference MW of OMP *S. aureus* can stimulate immune response among women of infertile couples.

1. Introduction

Infertility is a condition in which, for example, couples who have been married for a year making love two to three times a week without contraception do not become pregnant. The incidence of infertility is quite large and varies between 7% and 30% [1–6]. Infertility affects 10%–15% of women of childbearing age [7]. In 2005 and 2010, the Indonesian Central Bureau of Statistics reported that primary infertility was 8%, 12% and 12% in Indonesia, East Java and Mojokerto, respectively. Female-associated factors are responsible for 30%–40% of infertility and male-associated factors 60%–70% [2,7].

Vulvovaginitis is the most common gynecological condition encountered by primary care practitioners in women. The most common cause of symptomatic vaginitis in women (40%–45%) is nonspecific vaginitis [8]. Infections in developing countries play a role in 50%–80% of cases of infertility. The presence of immune reaction against spermatozoa contributes to as much as 2%–30% of infertility [9].

We explore bacterial wall constituent protein profiles that interact with immunoglobulin produced by infected women in infertile couples.

2. Materials and methods

The samples were all infertile couples in the outpatient department of Hasanah Muhammadiyah Hospital in Mojokerto, East Java, Indonesia from November 2012 to December 2013. The data of the infertile couples are complete (women's age,

*Corresponding author: Muhammad Anas, Doctorate Program of Biomedicine, Faculty of Medicine, Brawijaya University, Malang, Indonesia.
E-mail: muhanasjamil1@yahoo.co.id

Peer review under responsibility of Hainan Medical College.

[#] These authors contributed equally to this work.

marriage time, women's body weight, women's body height, education, menstrual history, semen analysis, hysterosalpingography, past medical history and past medication). The women in the infertile couples had not taken oral or vaginal antibiotic medication in the previous week.

All the women in these couples underwent some procedures to collect secretion from the uterine cervix and posterior fornix. To evaluate the bacterial colony an antibiotic sensitivity test of the uterine cervix and posterior fornix of the vagina was carried out. All the men in these couples were asked to collect semen after 4 d' abstinence before a semen analysis was performed.

2.1. Taking uterine cervix and vaginal fornix mucus

The vulva was cleaned with cotton wool moistened with normal sterile saline solution, avoiding the use of antiseptics, analgesics, and lubricants. The vagina was opened with a speculum that had been moistened/soaked in warm water until the uterine cervix appeared. Vaginal secretions were taken from the posterior fornix with a sterile swab, inserted directly into Stuart Transport Medium. Uterine cervix mucus was drained from the canal of the uterine cervix with a 3 cc sterile syringe without a needle, and inserted directly into Eppendorf tubes. After that, cervical secretions were taken from the ostium of the uterine cervix with a sterile swab, and inserted directly into Stuart Transport Medium.

2.2. Identification and bacterial culture

Bacteria were obtained from the vaginal swab samples and uterine cervix of women from infertile couples (primary and secondary) at the Infertility Clinic of Islam Hospital of Hasanah Muhammadiyah Mojokerto. Bacteria were grown on nutrient agar medium and incubated for 18–24 h at 37 °C. One colony was retrieved by using a loop, and Gram staining was performed followed by biochemistry tests, namely a catalase test and a coagulase test.

2.3. Antibiotics sensitivity test: Kirby–Bauer method

The inoculum was prepared using a sterile swab or three to five ose grabs for the same bacterial colonies and suspended in a tube containing a ±5 cc physiological sterile saline solution, then the used swab was disposed in a 2% hypochlorite solution. The bacterial suspension was compared with a McFarland turbidity standard of 0.5. Sterile cotton sticks were taken, dipped into the bacterial suspension and rotated several times, and then pressed on the wall of the tube. The cotton sticks were swapped evenly on the Mueller–Hinton agar surface while rotating the petri dish to 60°. The petri dish was closed, and left to stand for 3–5 min (no more than 15 min). Antibiotic discs were placed on the Mueller–Hinton agar surface. Discs were incubated at 35–37 °C for 16–24 h. The results of the sensitivity discs were read after 24 h. The antibiotics tested were ampicillin, amoxicillin, amoxycillin + clavulanat acid, gentamycin, amikacin, cotrimoxazole, fosfomycin, cefotaxime, ceftriaxone, imipenem, and ciprofloxacin.

2.4. Preparation, isolation, purification and profiling of Omp cell walls of bacteria

Nutrient broth seed results of bacteria were centrifuged at 4 °C at 6000 r/min for 10 min. Pellets containing bacterial cells were washed with PBS at a pH of 7.4. Pellets were suspended in a solution of glucose-EDTA-Tris pH 8 containing lysozyme 4 mg/mL and incubated at room temperature for 15 min. Pellet mix-GET-lysozyme solution was centrifuged at 4 °C at 12 000 *g* for 15 min. Supernatant containing the cell wall material was transferred, followed by purification by the salting-out method. Its concentration was measured with Nanodrop nd-1000, then its protein molecular weight profiled with SDS-PAGE [10,11].

3. Results

All women of infertile couples were still at an ideal age to conceive (28.1 ± 3.8) and time marriage mean is 4 years. Overall, the women of the infertile couples had a normal body weight, and only four women were overweight. Eighty-four percent of the infertile couples had primary infertility and 16% had secondary infertility (Table 1).

Clinical data from each infertile couple consisted of the condition of vaginal secretion, tubal patency test, semen analysis and outcome of infertility as listed (Table 2).

Bacterial cultures from uterine cervix and posterior fornix secretions from the vaginas of 19 women from infertile couples revealed the growth of 15 bacteria, three candidas and one no growth. Bacterial growth was dominated by (21%,4/19) *Staphylococcus aureus*, (21%,4/19) *Escherichia coli*, (16%,3/19) *Staphylococcus epidermidis*, (16,3/19) *Staphylococcus faecalis*, (11,2/19) *Streptococcus α-haemolyticus*, (11,2/19) *Candida albicans* and (5,1/19) no growth. The results of the antibiotic test for each bacteria are listed in Table 2. Western blot examination results for the ten types of OMP bacteria isolated from the samples are shown in Figure 1.

4. Discussion

In Table 1 it is shown that in terms of age, the husbands and wives were still at a healthy reproductive life stage, *i.e.* 28.1 ± 3.8 years. The highest age recorded was 36 years. Fritz and Speroff [7] found that the limit of the age factor affecting fertility in women started at more than 35 years [7]. The mean time married was 4.0 ± 2.2 years, but there was an extreme time of 11 years. In couples who handled the late state of infertility may occur due to the age of marriage is still

Table 1
Demographic data nonspecific vaginitis in women of infertile couple.

Parameters	Mean ± SD	Min–max
Age (husband)	32.9 ± 4.8	(23–45)
Age (wife)	28.1 ± 3.8	(21–36)
Time to marriage	4.0 ± 2.2	(1–11)
Body weight	51.5 ± 6.8	(34–66)
Body height	154.0 ± 3.8	(146–165)
BMI	21.7 ± 3.1	(15.5–28.6)
Education	**Husband**	**Wife**
Elementary school	1 (5%)	0 (0%)
Junior high school	3 (16%)	2 (11%)
Senior high school	9 (47%)	8 (42%)
Diploma	2 (11%)	3 (16%)
Graduate	4 (21%)	6 (32%)
Infertility	Primary	16 (84%)
	Secondary	3 (16%)

Table 2
Profile of bacterial isolates and antibiotic sensitivity test.

Sample	Name of isolates	Name of antibiotics												
		AMP	AML	AMC	GN	CTX	CRO	75	SXT	TE	C	FOS	CIP	Da
M6	*E. coli*	R	R	R	R	R	R	S	R	R	R	I	S	S
M4	*E. coli*	S	S	S	I	I	S	I	I	I	S	S	I	S
M3	*E. coli*	R	R	R	R	S	S	S	R	R	R	I	S	S
M1	*E. coli*	R	R	S	I	S	S	S	R	R	R	I	I	S
M9	*S. aureus*	S	S	S	I	I	I	S	R	R	R	I	S	S
M2	*S. aureus*	S	S	I	S	S	S	I	S	I	S	S	S	S
M19	*S. aureus*	R	R	I	S	R	R	I	R	R	R	S	R	I
M10	*S. aureus*	S	S	S	S	S	S	I	S	S	I	S	S	S
M8	*S. epidermidis*	I	I	S	I	S	S	S	S	R	R	I	S	S
M7	*S. epidermidis*	I	I	I	S	I	I	S	S	S	I	S	S	S
M18	*S. epidermidis*	R	R	R	R	R	R	R	R	R	R	I	R	R
M5	*S. fecalis*	S	S	S	I	S	S	S	S	R	R	I	S	S
M12	*S. fecalis*	S	S	S	I	S	S	S	S	R	R	I	S	S
M13	*Streptococcus α-haemolyticus*	S	S	S	S	S	S	S	S	I	S	I	S	I
M11	*Streptococcus α-haemolyticus*	S	S	I	R	S	S	S	S	S	S	S	S	S

AMP: ampicillin; AML: amoxicillin; AMC: amoxicillin + clavulanat; GN: gentamycin; CTX: cefotaxime; CRO: ceftriaxone; 75: cefoperazone; SXT: cotrimoxazole; Te: tetraxyclin; C: chloramphenicol; FOS: fosfomycin; Cip: ciprofloxacin; Da: clindamycin; R: resistance; S: sensitive; I: In termediate sensitive.

relatively young [7]. The proportion of the female body of infertile couples still within the normal range of 21.7 ± 3.1 that infertility disorders originate from abnormalities of GnRH hypothalamus minimal [7]. The most fertile couples are educated only 2% low education is junior to the bottom; this is a good indicator of the importance of being aware that help is needed because of the condition of infertility [7]. The incidence of primary infertility in this study was (84%,16/19) compared with secondary infertility (16%,3/19). This is different from the data obtained by Dhont *et al*. [12], which states that the greater incidence of secondary infertility is due to a lack of sterility in the internal genital organs and post-partum women [13].

In Table 2, it is shown that most couples who are infertile (47%,9/19) (M1, M2, M3, M5, M7, M9, M12, M13, M17) find it difficult to get pregnant because there are organic abnormalities in the analysis of semen and hysterosalpingography results. In patients with patent bilateral tubal patency condition (84%,16/19) or unilateral (16%,3/19), pregnancy is still possible because sperm can still be met by the ovum, but others that have organic abnormalities (19%,3/16) find it very difficult or even impossible to become pregnant. With a normal semen analysis (37%,7/19), abnormalities in the one to two parameter range (37%,7/19) or abnormal sperm with two parameters it is still possible to get pregnant. As for sperm abnormalities with more than two parameters (5%,1/19) or azoospermia (21%,4/19), it is almost or completely impossible to conceive naturally. Semen analysis showed that 21% could not impregnate, 5% found it difficult to impregnate, 37% found it possible to impregnate and 37% found it easy to impregnate.

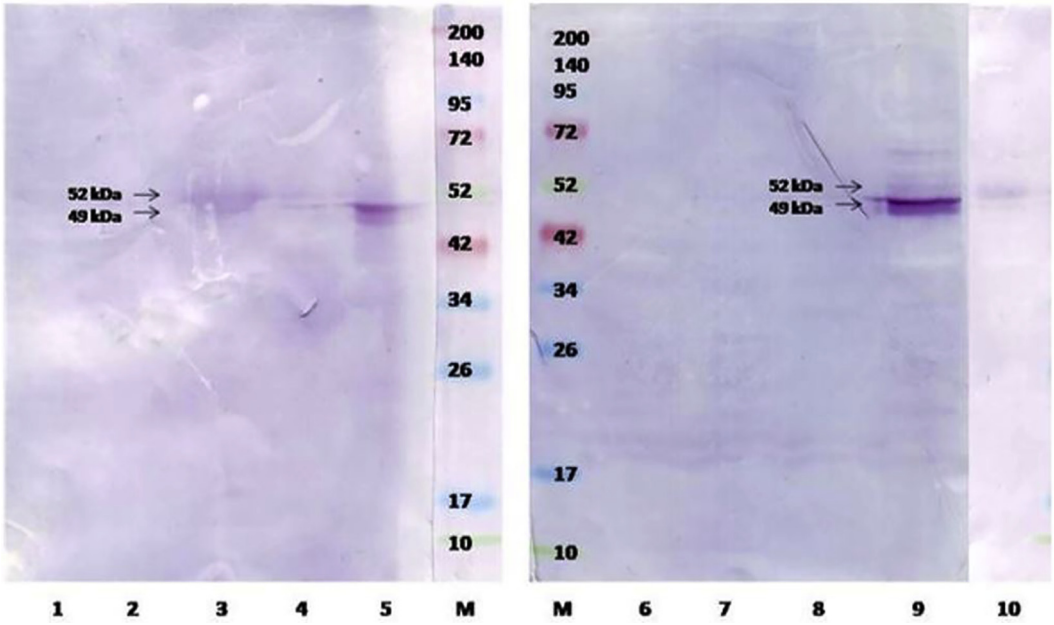


Figure 1. The results of western blot of bacterial sample OMP.
Lane 1: OMP *Streptococcus α-haemolyticus* M1, lane 2: OMP *E. Coli* M4, lane 3: OMP *S. aureus* M10, lane 4: OMP *S. epidermidis* M18 and lane 5: OMP *S. aureus* M19, which blotted with s-IgA cervix uteri M10, respectively. Lane 6: OMP *E. coli* M4, lane 7: OMP *Streptococcus α-haemolyticus* M11, lane 8: OMP *S. epidermidis* M18, lane 9: OMP *S. aureus* M19 and lane 10: OMP *S. aureus* M10, which blotted with s-IgA cervix uteri M19. M: marker.

Table 2 presents other data that can be found in the case of vaginal discharge/infection in infertile couples (63%,12/19). These results show almost no differences (80%) from those obtained by others [9] Almost all women of infertile couples found the growth of bacteria or fungi (95%,18/19) either with symptoms of vaginal discharge or not. This object caused by the vulvovagina is an area that is usually occupied by normal flora. Bacterial or fungal isolates obtained from the study classified as a potential pathogen. Isolation of aerobic bacteria was not assessed. One of the 19 patients (5%) did not reveal any bacterial growth. These results could be due to the time of transport. The bacteria obtained were dominated by *S. aureus* (27%,4/19) and *E. coli* (27%,4/19), and the rest comprised a group of bacteria *Enterobacteriaceae* others. This was similar to the findings of Sri Winarsih, with 51% *Staphylococcus* spp. (15% *S. aureus* and 36% *S. epidermidis*), 42% gram-negative rods and 7% *Streptococcus* spp. According to the results of analysis of sensitivity to antibiotics, the bacteria *E. coli*, *S. aureus* and *S. epidermidis* are resistant to common antibiotics, while *S. faecalis* and *Streptococcus α-haemolyticus* are still sensitive.

From isolated bacteria, clinical data were obtained on each of the infertile couples so we pay more attention to the bacteria *S. aureus* (M10 and M19), *S. epidermidis* (M18), *E. coli* (M4) and *Streptococcus α-haemolyticus* (M11). The subject is still possible to get pregnant and has been pregnant for *S. aureus* (M10). Western blot examination was carried out using OMP bacteria as antigens M4, M10, M11, M18 and M19 with s-IgA derived from the uterine cervix samples M10 and M19, and the results show that OMP M10 and M 19 which responded by cervical secretions M10 *S. aureus* (pregnant) and M19 *S. aureus* (unpregnant) indicate the protein bands MW 52 kDa and MW 49 kDa, while the protein bands which responded to s-IgA cervical secretions M19 (not pregnant) showed MW 52 kDa and MW 49 kDa, respectively. Both OMP *S. aureus* M10 and M19 show consistency similar to s-IgA M10 and M19, that OMP recognized, in particular, MW OMP *S. aureus* M19 and recognized strongly by both s-IgA M10 and M19. The introduction of s-IgA uterine cervix secretions against OMP *S. aureus* M10 and M19 is more specific because other bacteria *S. epidermidis* (M18), *E. coli* (M4) and *Streptococcus α-haemolyticus* (M11) are not recognized by s-IgA uterine cervix secretions of M10 and M19. This happens because of the maturation of immunoglobulin IgM and IgG become IgA then s-IgA are stimulated by repeated antigen exposure through the process of somatic hypermutation [14]. In particular, *S. aureus* M10 and M19 have different antibiotic sensitivity test results where *S. aureus* M10 is more sensitive and *S. aureus* M19 more resistant. Perhaps these results have a connection to the immunogenicity of their protein.

Based on the results of this study to determine the fertility disorder in infertile couples with the women of infertile couples who suffer nonspecific vaginitis due to *S. aureus*, further research needs to be developed on the existence of cross reaction between *S. aureus* bacteria and male spermatozoa of the infertile partner.

One of the bacteria species *S. aureus* that infects the vagina and causes nonspecific vaginitis can provoke an adaptive immune response by producing s-IgA. Difference MW of OMP *S. aureus* can stimulate immune response among women of infertile couples.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgement

The authors thank to Medical Faculty, Brawijaya University and Islam Hospital of Hasanah Muhammadiyah Mojokerto for facilitating this study.

References

[1] Jarow JP, Sigman M, Kolettis PN, Lipshultz LI, McClure RD, Nangia AK, et al. *The optimal evaluation of the infertile male: AUA best practice statement, revised 2010*. Maryland: American Urological Association Educations and Research, Inc.; 2010.
[2] Kamel RM. Management of the infertile couple: an evidence based protocol. *Reprod Biol Endocrinol* 2010; **8**: 21.
[3] Omoaregha JO, James BO, Lawani AO, Morakinyo O, Olotu OS. Psychosocial characteristics of female infertility in a tertiary health institution in Nigeria. *Ann Afr Med* 2011; **10**(1): 19-24.
[4] Philippov OS, Radionchenko AA, Bolotova VP, Voronovskaya NI, Potemkina TV. Estimation of the prevalence and causes of infertility in Western Siberia. *Bull World Health Organ* 1998; **76**(2): 183-187.
[5] Rabiu KA, Adewunmi AA, Akinlusi FM, Akinola OI. Female reproductive tract infections: understandings and care seeking behavior among women of reproductive age in Lagos, Nigeria. *BMC Womens Health* 2010; **10**: 8.
[6] Liu QZ, Zhu WJ, Liu HP, Jiang H. Evaluation on antisperm antibody in infertile women with chronic salpingitis. *J Reprod Contracep* 2014; **25**(3): 159-163.
[7] Fritz MA, Speroff L. *Clinical gynecology endocrinology and infertility*. 8th ed. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2011.
[8] Gor HB. Vaginitis. Available at: http://emedicine.medscape.com/article/257141-overview; 2012 [Accessed February, 2012]
[9] Krause WKH, Naz RK. *Immune infertility*. Berlin, Heidelberg: Springer Verlag; 2009.
[10] Laemli UK. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680-685.
[11] van Schilfgaarde M, van Ulsen P, Eijk P, Brand M, Stam M, Kouame J, et al. Characterization of adherence of nontypeable *Haemophilus influenzae* to human epithelial cells. *Infect Immun* 2000; **68**(8): 4658-4665.
[12] Dhont N, Luchers S, Muvunyi C, Vyankandondera J, De Naeyer L, Temmerman M, et al. The risk factor profile of women with secondary infertility: an unmatched case–control study in Kigali, Rwanda. *BMC Womens Health* 2011; **11**: 32.
[13] Harlow E, Lane D. *Antibodies: a laboratory manual*. New York: Cold Spring Harbor Laboratory; 1988, p. 386.
[14] Abbas AK, Lichtman AH, Pillai S. *Cellular and molecular immunology*. 7th ed. Philadelphia: Elsevier Saunders; 2012.