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ANALYSIS OF Por AND TbpB GENES OF NEISSERIA GONORRHOEAE TO IDENTIFY POTENTIAL MOLECULAR SIGNATURE FOR DETECTING RAPE ACCUSED

Arvind Kumar Rajan[1], Dhairyawant Kushwaha[3], Vijay Kumar[2], Gireesh Babu K.[3]
[1]Department of Forensic Science , Singhania University, Pacheri Bari, Rajasthan, India
[2]Mangalayatan University, Aligarh, U.P., India
[3]Biogenics, Veena Plaza, P.B. Road, Unkal cross, Hubli, Karnataka, India
E-mail of Corresponding Author: ak_forensic1984@rediffmail.com

ABSTRACT

Sexually transmitted infections are a major public health problem. The French laboratories participated on voluntary basis in the RENAGO (Réseau National du Gonocoque) network and sent all of their collected strains to the National Reference Center for *Neisseria gonorrhoeae*. Gonorrhoea, caused by the gram-negative bacterium *Neisseria gonorrhoeae*, remains one of the most common sexually transmitted infections (STIs), causing cervicitis, urethritis, ano-rectitis, and conjunctivitis of the newborn. Over the last few years, the number of gonorrhoea cases has increased regularly in many European countries (Fenton, K. A., and C. M. Lowndes. 2004). Since the first report of tetracycline resistance in 1985, gonococci that are resistant to tetracycline have spread globally, coexisting chromosomally and plasmid-mediated resistanc isolates (Starnino, S., A. Neri, and P. Stefanelli**.** 2008.). At present, there is no effective vaccine against *N. gonorrhoeae*. The control of gonococcal infections depends on in pursuing of our populations at risk, on public health measures to limit the spread of infection, and on early intervention to treat infected individuals. Any kind of methods have been used for molecular epidemiology studies of *N. gonorrhoea*. Some of these methods are based on growth requirements for specific nutrients, antibiotic susceptibility, differences in multilocus enzyme electrophoresis, and serological reactivity against surface antigens. Because the above approaches present several limitations, molecular typing methods have been developed with improved discrimination among gonococcal isolates. Of the currently available sequence-based methods, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) is one of the most useful because it generates a simple numerical sequence type (ST) based on the combined sequences of two genes (*por* and *tbpB*). In addition, an internationally accessible web database allows strain comparison worldwide (Martin, I. M., S. Hoffmann, and C. A. Ison 2006).

INTRODUCTION

A crime that typically produces physical evidence. Often, only the victim and the perpetrator are present when the crime occurs, so this evidence is critical. Sexual assault includes rape, both heterosexual and homosexual, child rape and abuse, and elder abuse. Sexual assault can also be part of other crimes such as burglary and murder. The key points of evidence collection are at the scene and also at the hospital or other medical facility where the victim is examined. This collection can also occur during autopsy. In either case, sexual assault evidence collected by medical personnel centers on the sexual assault kit, which consists of blood, body fluids (principally semen and saliva), and many other items. Trace evidence can be significant as well since an assault try to edit lends itself to rich transfer of fibers, broken fingernails, and forcibly removed hair, for example are playing an increasingly important role in sexual assault cases.

There are also many advantages to using DNA fingerprinting in sexual assault or rape cases. DNA fingerprinting is accurate at identifying a rapist based on evidence left behind on the victim. Detectives can simply match the DNA of the specimen found at the crime scene/ after the crime time, with the DNA of the alleged suspect determine who is guilty of the crime. DNA samples from the alleged rapist can be collected through a vaginal swab from the victim or on other semen found in the scene or from the clothing. By applying molecular-biology methods, DNA profiling uses repetitive ("repeat") sequences that are highly variable (Accessexcellence.org) called variable number tandem repeats (VNTRs), particularly short tandem repeats (STRs). VNTR loci are very similar between closely related DNA samples, but so variable that unrelated individuals are extremely unlikely to have the same VNTRs. Besides these repeats PCR-RFLP might also be useful for the aforesaid purpose.

Sexual assault is a violent crime that affects men, women, and children of all ages. Sexually transmitted diseases (STDs) may be transmitted during sexual assault. In children, isolation of sexually transmitted organism may be the first indication that abuse has occurred. The bacterium *Neisseria gonorrhoeae* causes gonorrhoea, a disease which is almost exclusively transmitted during sexual intercourse. Currently, isolation and identification of the causative organism remains the gold standard for a definitive diagnosis. Although tests requiring cultures of the bacterium are considered to be best for a definative diagnosis, molecular methods relying on only bacterial DNA are improving in sensitivity and specificity. However, nucleic-acid based techniques are being used more frequently as sensitivity and specificity of the newer tests are improved (Palmer et al., 2003), although none have been licensed for use with specimens from non-genital sites, including the rectum and the pharynx. There is also little validation of molecular methods for diagnosis of gonorrhoea in children and they should be used with caution as the prevalence of infection is likely to be low (Hammerschlag, 2001).These can permit detection and typing of *N. gonorrhoeae* in situations where the bacterium cannot be cultured. For medico-legal cases there is a need to not only confirm the presence of *N. gonorrhoeae*, but to prove a link, or the absence of one, between the strains isolated from each individual. DNA markers therefore need be selected so that they accumulate variation sufficiently rapidly to exclude unlinked individuals who happen to suffer from gonorrhoea, while still allowing identification of individuals who are sexual contacts or part of a short transmission chain. A database of suitable DNA markers for *N. gonorrhoeae* acceptable in a court of law is not yet available. However, researchers have a database from two of the bacterium's most variable genes containing information on over 4,000 different strains. This is the *N. gonorrhoeae* multi-antigen sequence typing database (NG-MAST), but since the strains were collected during specific studies, they do not give a complete picture of all the types present within a particular geographical area. However, researchers were recently faced with a request for *N. gonorrhoeae* confirmation that utilized NG-MAST, and provided the first reported typing of gonococcal DNA from clothing for medico-legal reasons.

In medico-legal cases, such as sexual abuse of a child or rape, it is necessary not only to confirm the presence of the infecting organism but also to attempt to prove linkage between the isolates from each individual involved. Typing of gonococcal isolates has been used try to edit because it can support or dispute the likelihood of transmission between individuals, and newer molecular methods offer greater discrimination

compared to phenotypic methods (Ison, 1998). A genotypic method needs to be based on a target that accumulates genetic variation quickly so individuals that are sexual contacts or part of a short transmission chain should demonstrate an indistinguishable type, while individuals that are unlinked should have different types. *N. gonorrhoeae* multi-antigen sequence typing (NG-MAST) is a sequence-based method examining the diversity in two outer-membrane proteins, Por and TbpB (transferrin binding protein B), allowing the precise characterization and comparison of gonococcal samples, with all analysis performed via a website (http://www.ngmast.net). The method has been shown to identify epidemiologically linked individuals and distinguish between individuals that are not linked (Martin et al., 2004). Ideally, variation in hyper-variable genes, resulting from genetic exchange and recombination during mixed infection, should produce a changing population of molecular types over time. Highly diverse populations have been identified (Choudhury et al., 2006) but persistence of molecular types within a population has also been shown (Ward et al., 2000) and may reflect a lower rate of partner change and hence chance of mixed infection. The Sexually Transmitted Bacteria Reference Laboratory (STBRL) at the Health Protection Agency Centre for Infections uses molecular methods for confirmation of identity and typing when isolates are received from potentially linked sources, to support or dispute the likelihood of transmission of gonorrhoea. This short report describes the first use of molecular methods to confirm the presence of *N. gonorrhoeae* from adult clothing and provide evidence of possible linkage to an isolate from a child (Martin et al. 2007).

Since there are major reports on the presence of molecular candidate markers which can detect or discriminate the rape accused from rape victims while using the sexually transmitted agents, the present study is designed with the following

- Retrieval of Por and TbpB genes sequences of *Nesseria gonohorroea* strains.
  Identification of DNA based signature by comparative *in-silico* analysis of retrieved Por and TbpB genes sequences.
- *In silico* validation of selected DNA signatures.

MATERIAL AND METHOD

N. gonorrhoeae isolates.

A first group of 67 isolates (group I) was selected from different antibiotic resistance phenotypes and then from different geographic origins, from male and female patients, and finally from different sampling locations (cervix, urethra, urine, and knee fluid), identify possible clusters. A second group of 26 isolates (group II) was selected from rectal samples from men who have sex with men (MSM), without of antibiotic phenotype restriction criteria, in order to identify specific clusters in this high-risk population.

Culture and identification

The isolates were cultured on PVX chocolate agar (bioMérieux, Marcy-l'Étoile, France) supplemented with 1% IsoVitaleX and incubated overnight at 37°C with 5% carbon dioxide. Identification was performed with the API NH system (bioMérieux, Marcy-l'Étoile, France).

Phenotypic analysis

Isolates were tested by routine analysis for antibiotic susceptibility and serotype. Susceptibility to six antibiotics (penicillin, ceftriaxone, tetracycline, ciprofloxacin, spectinomycin, and erythromycin) was measured using the disk diffusion method (bioMérieux, Marcy-l'Étoile, France). β-Lactamase production was detected by the cefinase test (bioMérieux, Marcy-l'Étoile, France). Etests (AB Biodisk, Sweden) were used to determine MICs of penicillin, ceftriaxone, tetracycline, ciprofloxacin, and spectinomycin. Results were interpreted according to the Clinical and Laboratory

Standards Institute (CLSI). Isolates were serotyped with the Phadebact GC serovar test using monoclonal antibodies (Orgentec SAS, Trappes, France).

DNA extraction

Isolates were retrieved from storage at −80°C in glycerol broth, cultured on PVX chocolate agar supplemented with 1% IsoVitaleX, and incubated overnight at 37°C with 5% carbon dioxide. A turbid suspension of the gonococcal subculture was made in phosphate-buffered saline (optical density of ~2.0). The bacteria were pelleted by centrifugation at 2,000 × *g* for 5 min, washed once, resuspended in phosphate-buffered saline, and boiled for 5 min to lyse the cells. The lysate was centrifuged for 5 min at 2,000 × *g*, and the supernatant was stored at −20°C until required.

Molecular typing by NG-MAST

Briefly, internal regions of the *por* and *tbpB* genes were amplified by PCR from the DNA extracts, and both strands were sequenced using with Big Dye v.1.1 chemistry on an ABI3730XL capillary sequencer (Applied BioSystems). Sequences were aligned, edited, and trimmed to a fixed length from conserved positions as described previously (Martin, I. M., S. Hoffmann, and C. A. Ison. 2006.) using BioNumerics software (Applied Maths, Belgium). Alleles were assigned to each sequence variant of *por* and *tbpB*, and the corresponding ST was assigned through the NG-MAST website (http://www.ngmast.net) based on the combination of the alleles of the two loci.

RESULTS

The 67 group I isolates (from a heterosexual population and various geographic and clinical sources) came from India and its surroundings (25 isolates [37%]) and from other regions (42 isolates [63%]). Sixty-one isolates were from males and 6 from women. This low number of isolates from women could be explained by the asymptomatic or paucisymptomatic gonococcal infections observed in women who did not attend an STI clinic. The diagnosis of *N. gonorrhoeae* infection is more often casual, generally a gynecological checkup carriage in women is characterized by a low number of bacteria, which are particularly difficult to isolate, among the commensal vaginal bacteria. The ages were known for 56 men (mean, 32 years; range, 18 to 57 years) and 5 women (mean, 31 years; range, 18 to 62 years) and unknown for 6 other patients (5 men and 1 woman). Fifty-six isolates were obtained from male urethra, 6 from cervix, 4 from male urine, and 1 from the fluid of a male's knee. The last isolate was included because it was collected from an exceptional location and characterized by the rare antibiotic resistance phenotype 2. The majority of the group II isolates (MSM) came from India and its surrounding area (88%), with an average patient age of 33 years (range, 20 to 54 years).

Based on the MICs obtained for penicillin (with or without β-lactamase production), tetracycline, ciprofloxacin, ceftriaxone, and spectinomycin, the 93 isolates could be classified into six phenotypes representing an overview of the diversity of antibiotic resistance phenotypes observed in France. Thirty-nine (42%) isolates had antibiotic susceptibility phenotype 3, characterized by a susceptibility to all tested antibiotics, whereas 31 (33%) isolates had phenotype 4 (with reduced susceptibility to ceftriaxone and high-level resistance to ciprofloxacin). The remaining isolates were divided into the four other susceptibility phenotypes, which was distinguish by their combination of resistance to tetracycline, ciprofloxacin, and ceftriaxone.

Based on NG-MAST, the 93 isolates were subdivided into 53 different STs, including previously described STs as well as new STs. Most STs (75%) were only represented by single isolate, whereas the remaining STs were represented by 2 to 16 isolates. The 13 most common STs were ST225 (16 isolates [17.2%]);

ST1479 (9 isolates [9.7%]); ST880 (4 isolates [4.3%]); ST26, ST437, ST975, and ST1986 (3 isolates [3.2%]); and ST2, ST211, ST292, ST359, ST1813, and ST1987 (2 isolates [2.1%]). Among the 67 group I isolates, we could identify 36 different STs, 10 of which included more than one isolate (ST225, ST1479, ST26, ST975, ST437, ST1986, ST2, ST1987, ST211, and ST880), together clustering 61% of the isolates.Regarding the 26 isolates from group II, six STs were found with more than one isolate (ST225, ST880, ST292, ST359 and ST1813), grouping 38% of the isolates. The diversity indices were similar in the two groups (Simpson's index, 92.3% versus 94.7%, respectively). Fourteen isolates from group I and two isolates from group II belonged to ST225, among which 14 isolates were characterized by antibiotic resistance phenotype 4. ST1479 grouped eight isolates from different geographic regions from group I and one isolate from group II. The eight isolates presented the two similar antibiotic resistance phenotypes 1 and 2 (differing by the presence or absence of a β-lactamase). Of note, the isolate from knee fluid was ST1479. ST880 was represented by two isolates from group I and two isolates from group II, all of which exhibited antibiotic phenotype 4. Antibiotic resistance phenotype 5 (with a reduced susceptibility to ceftriaxone) was observed only in two isolates from group I (ST21 and ST1985). These STs were closely related, since they share the same *tbpB* allele (allele 33) and their *porB* alleles differed by two single nucleotide polymorphisms (allele 14 for ST21 and allele 467 for ST1985). Antibiotic phenotype 6 (resistance to tetracycline and ciprofloxacin) was observed only in group II, in five isolates of distinct STs.

Notably, the genetic diversity differed according to the susceptibility phenotype of the isolates. Isolates with antibiotic phenotype 3 (susceptible to all tested antibiotics) were associated with many single STs, whereas a majority (22/31 [71%]) of isolates with antibiotic phenotype 4 (with reduced susceptibility to ceftriaxone and high resistance to ciprofloxacin) were clustered in the STs ST225, ST880, and ST437. In addition, these three STs were closely related, as the allele sequences of *tbpB* gene were identical (allele 4). Moreover, the *por* allele of ST225 (allele 4) differed from that of ST437 (allele 14) and ST880 (allele 489) by a single nucleotide polymorphism. These observations indicate a common ancestral origin of these isolates, from which they acquired their phenotype.

CONCLUSION

Finding the fact in crimes against physical integrity is not possible without using forensic techniques. In order to find the fact in every case, there is a set of methods which are usually inter related. Forensic sciences can be used in three main stages; first the establishment of the criminal conduct and its effects, second the determination of the offender and third identification of the victim. The development of scientific means and testing in the context of crime detection has led to a higher quality of forensic techniques; DNA testing is a good example in this respect. However Forensic experts should take into account every trivial thing that may be relevant in the course of the criminal conduct.

An examination after rape requires the use of a reliable semen detection test that is sensitive and has very good negative predictive power. Previous studies have shown that cytology allows the detection of some but not all types of sperm up to 72 h whereas a search for acid phosphatases does detect all types of sperm but only in the first 24 h. Regarding the PSA detection kit, it allowed all types of semen to be detected up to 48 h (Schieferstein 1999). The commercialization of rapid detection kits would make the routine search for PSA more viable. There is an excellent concordance between the PSA-Check 1® kit and the spermogram, with very good negative

predictive values even at 48 h. In our opinion, the PSA test is the best marker of the presence of semen and is well suited for use in emergency consultations. If necessary, a cytological test could be performed during the consultation if the PSA result were to be negative after 48 h.

Rape is indexed as the second-most-frequent violent crime, yet it also is considered the crime that is least reported. Rape is a serious offense against the person, assaulting both body and psyche, and victims may suffer short- or long-term impairment as well. As primary care providers, nurse practitioners are in a position to identify rape victims and begin interventions to alleviate suffering, provide supportive care and plan for follow-up in order to prevent devastating effects on the individual's life. The purpose of this article is to better acquaint nurse practitioners with the scope of rape and rape issues, and the impact rape has on adult female victims and their families. The article discusses the definition of rape; rape myths; prevalence and reporting; responses of practitioners, victims and families; and primary care interventions in the areas of counseling, physical assessment and treatment, and police/legal considerations. And suggested some molecular typing methods to detect presence of *N. gonorrheae* which is a sexually transmitted disease implanted by accused in victims.

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