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Phenotypic and genetic characterisation of bacterial sexually transmitted infections in Bissau, Guinea-Bissau, West Africa: a prospective cohort study

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ABSTRACT

Background: Knowledge regarding characteristics and transmission of *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Mycoplasma genitalium* and antibiotic resistance in *N gonorrhoeae* in Guinea-Bissau, West Africa, is entirely lacking.

Objectives: To characterise *N gonorrhoeae*, *C trachomatis* and *M genitalium* samples from Guinea-Bissau and to define bacterial populations, possible transmission chains and for *N gonorrhoeae* spread of antibiotic-resistant isolates.

Design: Prospective cohort study.

Setting: Two sexual health and family planning clinics, Bissau, Guinea-Bissau.

Participants: Positive samples from 711 women and 27 men.

Material and methods: Positive samples for *N gonorrhoeae* (n=31), *C trachomatis* (n=60) and *M genitalium* (n=30) were examined. The gonococcal isolates were characterised with antibiograms, serovar determination and *N gonorrhoeae* multiantigen sequence typing (NG-MAST). The *C trachomatis ompA* gene and the *M genitalium mgpB* gene were sequenced, and phylogenetic analyses were performed.

Results: For *N gonorrhoeae*, the levels of resistance (intermediate susceptibility) to ciprofloxacin, erythromycin, rifampicin, ampicillin, tetracycline, penicillin G and cefuroxime were 10% (0%), 6% (10%), 13% (10%), 68% (0%), 74% (0%), 68% (16%) and 0% (84%), respectively. All isolates were susceptible to cefixime, ceftriaxone, spectinomycin and azithromycin, and the minimum inhibitory concentrations of kanamycin (range: 8–32 mg/l) and gentamicin (range: 0.75–6 mg/l) were low (no resistance breakpoints exist for these antimicrobials). 19 NG-MAST sequence types (STs) (84% novel STs) were identified. Phylogenetic analysis of the *C trachomatis ompA* gene revealed genovar G as most prevalent (37%), followed by genovar D (19%). 23 *mgpB* STs were found among the *M genitalium* isolates, and 67% of isolates had unique STs.

Conclusions: The diversity among the sexually transmitted infection (STI) pathogens may be associated with suboptimal diagnostics, contact tracing, case reporting and epidemiological surveillance. In Guinea-Bissau, additional STI studies are vital to estimate the STI burden and form the basis for a national sexual health strategy for prevention, diagnosis and surveillance of STIs.

ARTICLE SUMMARY

Article focus

- Knowledge regarding characteristics and transmission of *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Mycoplasma genitalium* and antibiotic resistance in *N gonorrhoeae* in Guinea-Bissau, West Africa, is entirely lacking.
- We aimed to phenotypically and genetically characterise *N gonorrhoeae*, and genetically characterise *C trachomatis* and *M genitalium* samples from women attending two sexual health clinics in Bissau, Guinea-Bissau and to define the bacterial populations, possible transmission chains and for *N gonorrhoeae* also to define the presence and spread of antibiotic-resistant isolates from women and additionally a group of symptomatic men.

Key messages

- In Guinea-Bissau, *N gonorrhoeae* isolates displayed high level of resistance to traditional gonorrhoea antimicrobials but have remained susceptible to extended-spectrum cephalosporins, spectinomycin and azithromycin.
- Genovar G, D and F were the most prevalent *C trachomatis* genovars, the usually most common genovar among heterosexuals, that is, genovar E, was rare.
- The diversity among the bacterial STI pathogens may be associated with suboptimal diagnostics, contact tracing, case reporting and epidemiological surveillance. Additional studies are vital to estimate the STI burden and form the basis for a national sexual health strategy for prevention, diagnosis and surveillance.

INTRODUCTION

Guinea-Bissau, West Africa, is classified as the sixth least developed country in the world.[1]

ARTICLE SUMMARY

Strengths and limitations of this study

- This is the first time *N gonorrhoeae*, *C trachomatis* and *M genitalium* samples from Bissau, Guinea-Bissau, have been phenotypically and genetically characterised to define the bacterial populations, possible transmission chains and antibiotic resistance in *N gonorrhoeae*.
- The study sample was relatively small, and in this setting, it is difficult to perform appropriate sample transportation and storage, as well as optimised diagnostics, which may have resulted in that true-positive samples were lost.

The total number of inhabitants is about 1.6 million with 250 000–300 000 living in the capital city Bissau. The healthcare system is exceedingly limited and highly centralised to Bissau.

Guinea-Bissau has previously presented the highest HIV-2 prevalence globally. However, after the civil war in 1998–1999, the HIV-2 prevalence has decreased and in contrast the HIV-1 prevalence has increased.[2] HIV seropositive individuals have shown a higher prevalence of bacterial sexually transmitted infections (STIs), and presence of several bacterial STIs has been associated with a facilitated HIV acquisition and transmission.[2 3]

Present knowledge regarding the prevalence of bacterial STIs in Guinea-Bissau is highly limited. Studies from 2001 to 2002 described a prevalence of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infections of 17% and 4%, respectively, among women with vaginal discharge, and in pregnant women, the prevalence rate for *Mycoplasma genitalium* was 6.2%.[4 5] In a recent study of women attending two sexual health clinics in Bissau, due to urogenital problems, the prevalence of *N gonorrhoeae*, *C trachomatis* and *M genitalium* infections was 1.3%, 12.6% and 7.7%, respectively.[2]

In Guinea-Bissau, in routine, the laboratory diagnosis of *N gonorrhoeae* is mainly based on microscopy of Gram stained urogenital smears, no appropriate diagnostics is performed for *C trachomatis* and *M genitalium* and no previous study has phenotypically and genetically typed strains of these bacterial STIs. Consequently, knowledge regarding the characteristics and transmission of circulating strains of *N gonorrhoeae* (including antibiotic resistance), *C trachomatis* and *M genitalium* is entirely lacking.

The aims of the present study were to phenotypically and genetically characterise *N gonorrhoeae* and genetically characterise *C trachomatis* and *M genitalium* samples from women attending two sexual health clinics in Bissau, Guinea-Bissau and to define the bacterial populations, possible transmission chains and for *N gonorrhoeae* to define the presence and spread of antibiotic-resistant isolates from women and additionally a group of symptomatic men.

MATERIALS AND METHODS

Study population

The current study further examined specimens obtained in a previous prevalence study of 711 women attending two sexual health and family planning clinics (Aguibef Clinic and Centro Materno-Infantil Clinic) for urogenital problems in Bissau, Guinea-Bissau. All women visiting the clinics from February 2006 to January 2008 were offered participation.[2] Sixty samples analysed positive for *C trachomatis*, 30 analysed positive for *M genitalium* and four culture-positive *N gonorrhoeae* isolates were available for further examination. In addition, 27 *N gonorrhoeae* isolates cultured, during February 2006 to January 2008, from men (n=27) with urogenital symptoms at the National Public Health Laboratory, Bissau, Guinea-Bissau, were included.

Laboratory characterisation

All 31 *N gonorrhoeae* isolates were initially cultured and preserved at −70°C as previously described.[2 6] Briefly, endocervical (women) or urethral (men) samples were cultured within 5 h on modified Thayer–Martin medium for 48–72 h. Species confirmation was based on identification of rapid oxidase production, Gram–negative diplococci in microscopy and use of PhadeBact Monoclonal GC Test (Bactus, Stockholm, Sweden).[2] Antibiotic susceptibility testing, serovar determination, DNA isolation and genetic characterisation by means of *N gonorrhoeae* multiantigen sequence typing (NG-MAST) were performed as previously described.[7]

C trachomatis and *M genitalium* specimens were initially diagnosed with PCR as previously described.[2] Briefly, DNA was isolated from endocervical samples using the E.Z.N.A. Tissue DNA Kit (Omega Bio-tek, Doraville, Georgia, USA), and conventional PCRs, using previously described primers, were performed for diagnosis of *M genitalium* (target: *16S rRNA* gene and, for confirmation, MgPa adhesion (*mgpB*) gene) and *C trachomatis* (target: *ompA* gene).[2]

For typing of *C trachomatis*, the *ompA* gene was PCR amplified, the PCR product was purified and subsequently sequenced and finally about 1100 nucleotides of the *ompA* sequence was used in a phylogenetic analysis for determination of the genovar, as previously described.[8]

For typing of *M genitalium*, the *mgpB* gene was PCR amplified and sequenced as previously described,[9] with some modifications. These modifications included use of 0.4 µM of each of the primers MgPa-1 and MgPa-3 (Scandinavian Gene Synthesis AB, Köping, Sweden) and 2.0 U of *AmpliTaq* Gold DNA polymerase (PE Biosystems, Branchburg, New Jersey, USA). The PCR programme consisted of an *AmpliTaq* Gold DNA polymerase activation step at 94°C for 10 min, followed by 40 sequential cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min and finally an extension step of 72°C for 5 min. All PCR products were stored at 4°C prior to purification. The PCR products were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim, Indianapolis, Indiana, USA) according to the manufacturer's instructions. The PCR products were sequenced with the same primers as used in the PCR, utilising the ABI PRISM BigDye Terminator Cycle Sequencing Ready

Reaction v3.1 kit (Applied Biosystems, Warrington, UK) and an ABI Sequencer 3120 (Applied Biosystems), in accordance with the instructions from the manufacturer. Phylogenetic analysis was performed in the same way as for *C trachomatis*.[8]

RESULTS

Characterisation of *N gonorrhoeae*

The results of the antibiotic susceptibility testing of all the *N gonorrhoeae* isolates (n=31) are presented in table 1.

Briefly, the levels of resistance (intermediate susceptibility) to ciprofloxacin, erythromycin, rifampicin, ampicillin, tetracycline, penicillin G and cefuroxime were 10% (0%), 6% (10%), 13% (10%), 68% (0%), 74% (0%), 68% (16%) and 0% (84%), respectively. Twenty-one (68%) of the 31 isolates were β-lactamase producing. All isolates were susceptible to cefixime, ceftriaxone, spectinomycin and azithromycin, and the minimum inhibitory concentrations (MICs) of kanamycin (8–32 mg/l) and gentamicin (0.75–6 mg/l) were low (no resistance breakpoints exist for these antimicrobials) (table 1).

All the *N gonorrhoeae* isolates were serotypeable, 20 (64.5%) were determined as serogroup WII/III (PorB1b). These isolates were assigned four different serovars: Bropt (n=16), Bropst (n=2), Bpyvut (n=1) and Brpyvust (n=1). The remaining 11 (35.5%) isolates were determined as serogroup WI (PorB1a). These isolates were assigned two different Ph serovars: Arst (n=10) and Arost (n=1).

The isolates were assigned to 19 different NG-MAST sequence types (STs), of which 16 (84%) have not been previously described (table 2). The ST3176 (n=7), ST783 (n=5) and ST3182 (n=3) were the most prevalent STs. Of these, ST783 has been described in UK, while ST3176 and ST3182 were not earlier described. The remaining 16 isolates were all of different sequence types. Of these, ST1318 has been described in isolates from Arkhangelsk, Russia, Scotland and Canada, and ST2187 in Australia (NG-MAST website: http://www.ng-mast.net).

Notable, the three ciprofloxacin-resistant isolates (all displaying MIC≥1.5 mg/l) were assigned three different STs (ST3181, ST3184 and ST3186) and two different serovars (Bropst, n=2, and Brpyvust, n=1).

Characterisation of *C trachomatis*

Phylogenetic analysis of the *ompA* gene sequences from 60 *C trachomatis* samples revealed that the most frequent genovar was G (22 isolates, 37%), followed by D (19%), F (17%), I (14%), E (5%), J (5%), H (1.5%) and K (1.5%) (table 2). Accordingly, genovar E that is the most prevalent genovar followed by genovar F and genovar D in studies from other settings globally was relatively rare.[10–19]

Characterisation of *M genitalium*

A total of 23 *mgpB* STs were found among the 30 *M genitalium* samples. Only three clusters, one with four isolates ('ST1') and two containing three isolates ('ST2' and 'ST3'), were detected (figure 1, table 2). The remaining sequence types ('ST4'–'ST23') were only represented by single isolates.

DISCUSSION

In the present study, bacterial STIs in Guinea-Bissau were for the first time characterised, phenotypically and genetically, demonstrating highly divergent populations of *N gonorrhoeae* and *M genitalium*, and compared with other settings worldwide an unusual genovar distribution for *C trachomatis*. However, due to the limited number of samples examined, all results need to be interpreted with caution.

The genetic characterisation of the 31 *N gonorrhoeae* isolates showed a total of 19 different NG-MAST STs, and only three (16%) of these STs have been previously

Table 1 Antibiotic susceptibility of 31 *Neisseria gonorrhoeae* isolates from Bissau, Guinea-Bissau

Antibiotic (breakpoints)	Susceptible (%)	Intermediate (%)	Resistant (%)
Penicillin G (S≤0.064/R>1.0)*	5 (16)	5 (16)	21 (68)†
Ampicillin (S≤0.125/R>3.0)	10 (32)	0	21 (68)†
Cefixime (S≤0.125/R>0.125)*	31 (100)	0	0
Ceftriaxone (S≤0.125/R>0.125)*	31 (100)	0	0
Cefuroxime (S≤0.064/R>1.0)	5 (16)	26 (84)	0
Azithromycin (S≤0.25/R>0.5)*	31 (100)	0	0
Erythromycin (S≤0.25/R>0.5)	26 (84)	3 (10)	2 (6)
Ciprofloxacin (S≤0.032/R>0.064)*	28 (90)	0	3 (10)
Spectinomycin (S≤64/R>64)*	31 (100)	0	0
Tetracycline (S≤0.5/R>1.0)*	8 (26)	0	23 (74)
Rifampicin (S≤0.125/R>32)	24 (77)	3 (10)	4 (13)
Gentamicin	MIC range: 0.75–6 mg/l		
Kanamycin	MIC range: 8–32 mg/l		

*Breakpoints (for susceptible (S≤x mg/l) and resistant (R>y mg/l)) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org) were used, when available.
†All were β-lactamase producing.
MIC, minimum inhibitory concentration.

Table 2 Distribution of different genotypes of *Chlamydia trachomatis*, *Mycoplasma genitalium* and *Neisseria gonorrhoeae* among infected women in Guinea-Bissau

Chlamydia trachomatis **(genovar, n=60)***	***Mycoplasma genitalium*** **(sequential numbers, n=30)†**	***Neisseria gonorrhoeae*** **(NG-MAST, n=31)**
G (37%)	1 (13%)	ST3176 (23%)‡
D (19%)	2 (10%)	ST783 (16%)
F (17%)	3 (10%)	ST3182 (10%)‡
I (14%)	4–23 (single isolates 67%)	ST2187 (single isolate, 3%)
E (5%)		ST1318 (single isolate, 3%)
J (5%)		ST3177–81 (single isolates, 13%)‡
H (1.5%)		ST3183–89 (single isolates, 26%)‡
K (1.5%)		ST3376–77 (single isolates, 6%)‡

*Based on *ompA* genotyping.
†Based on sequencing of the *M genitalium* MgPa adhesion (*mgpB*) gene.
‡Novel NG-MAST STs.

reported, that is, ST783, ST1318 and ST2187 (NG-MAST website: http://www.ng-mast.net). Accordingly, ST783 has been described in UK; ST1318 in Russia,[20] Scotland and Canada and ST2187 in Australia (NG-MAST website: http://www.ng-mast.net). The ST3176 (n=7), ST783 (n=5) and ST3182 (n=3), were the most prevalent STs, while the remaining 16 STs were represented by single isolates. The high number of STs represented by single isolates could be a consequence of suboptimal diagnostics, limited and biased sampling, ineffective or non-existing contact tracing, local and recent emergence of new STs and import of strains.

The serological characterisation divided the isolates into 20 (64.5%) serogroup WII/III (PorB1b) isolates and 11 (35.5%) serogroup WI (PorB1a) isolates. The proportion of serogroup WI (PorB1a) isolates was relatively high compared with, for example, Sweden with 26% WI (PorB1a) isolates reported in 2009,[21] Italy with 4.1% WI (PorB1a) isolates in 2008[22] and Russia with 26% WI (PorB1a) isolates in 2007[20] but lower than described from India with 46.7% WI (PorB1a) isolates in 2007.[23] The isolates from Guinea-Bissau were further divided into six different Ph serovars.

As clearly shown, the genetic characterisation (19 NG-MAST STs) had a substantially higher discriminative power than the serovar determination (six serovars). Furthermore, no NG-MAST STs were further subdivided using serovars determination. Accordingly, genetic typing should preferably be used for epidemiological purposes, especially in geographical areas that comprise highly heterogeneous *N gonorrhoeae* populations.

The antibiotic susceptibility testing displayed relatively high levels of resistance and/or intermediate susceptibility to most antibiotics previously recommended as first line for treatment of gonorrhoea, for example, penicillins, tetracycline, erythromycin and cefuroxime. However, due to the low number of gonococcal isolates (n=31) examined in the present study, the resistance levels need to be interpreted with caution. The resistance level to ciprofloxacin (10%) was relatively low. Nevertheless, at the time of collection of the gonococcal isolates (2006–2008), similar low levels of resistance to ciprofloxacin were shown also in other studies from African countries, for example, from Malawi Mozambique, Central African Republic and Madagascar.[24–26] These ciprofloxacin resistance levels were substantially lower than the levels described from many other countries worldwide, where it is now a fear that gonorrhoea may become untreatable in certain circumstances.[27 28] However, more recent studies from African countries such as Kenya and South Africa have shown a rapidly increasing resistance to ciprofloxacin.[29 30] The slower pace of emerging ciprofloxacin resistance in Guinea-Bissau and several other African countries strains may reflect the fact that fluoroquinolones for treatment of gonorrhoea were not widely used as early in Guinea-Bissau (as well as several other African countries), as in other settings worldwide. Fortunately, all isolates were susceptible to cefixime, ceftriaxone, azithromycin and spectinomycin and had low MICs of gentamicin and kanamycin. Nevertheless, due to the high and increasing resistance levels to many of these antibiotics worldwide, it is essential to enhance the gonococcal antibiotic susceptibility surveillance in African countries as well as globally. In some settings, the resistance levels to previously recommended first-line antibiotics may have remained low and these antibiotics might be valuable to continue to use for treatment of gonorrhoea in these specific settings. Nevertheless, that type of recommendation needs to be supported by sufficient local, appropriate and quality-assured antibiotic susceptibility data.

The phylogenetic analysis of *ompA* gene sequences in the 60 *C trachomatis* samples revealed that genovar G (37%) was the most frequent genovar, followed by genovar D (19%) and genovar F (17%). Studies from other settings globally, from 2000 to 2011, have shown that genovar E is the most prevalent followed by genovar F and genovar D.[10–19] Furthermore, a longitudinal study over a 9-year period (1988–1996) in Seattle showed similar serovar distribution as reported from other parts of the world and also a stability in the serovar groups over time, except for the more rare serovars I and K.[11] Genovar G, which was the most frequent genovar

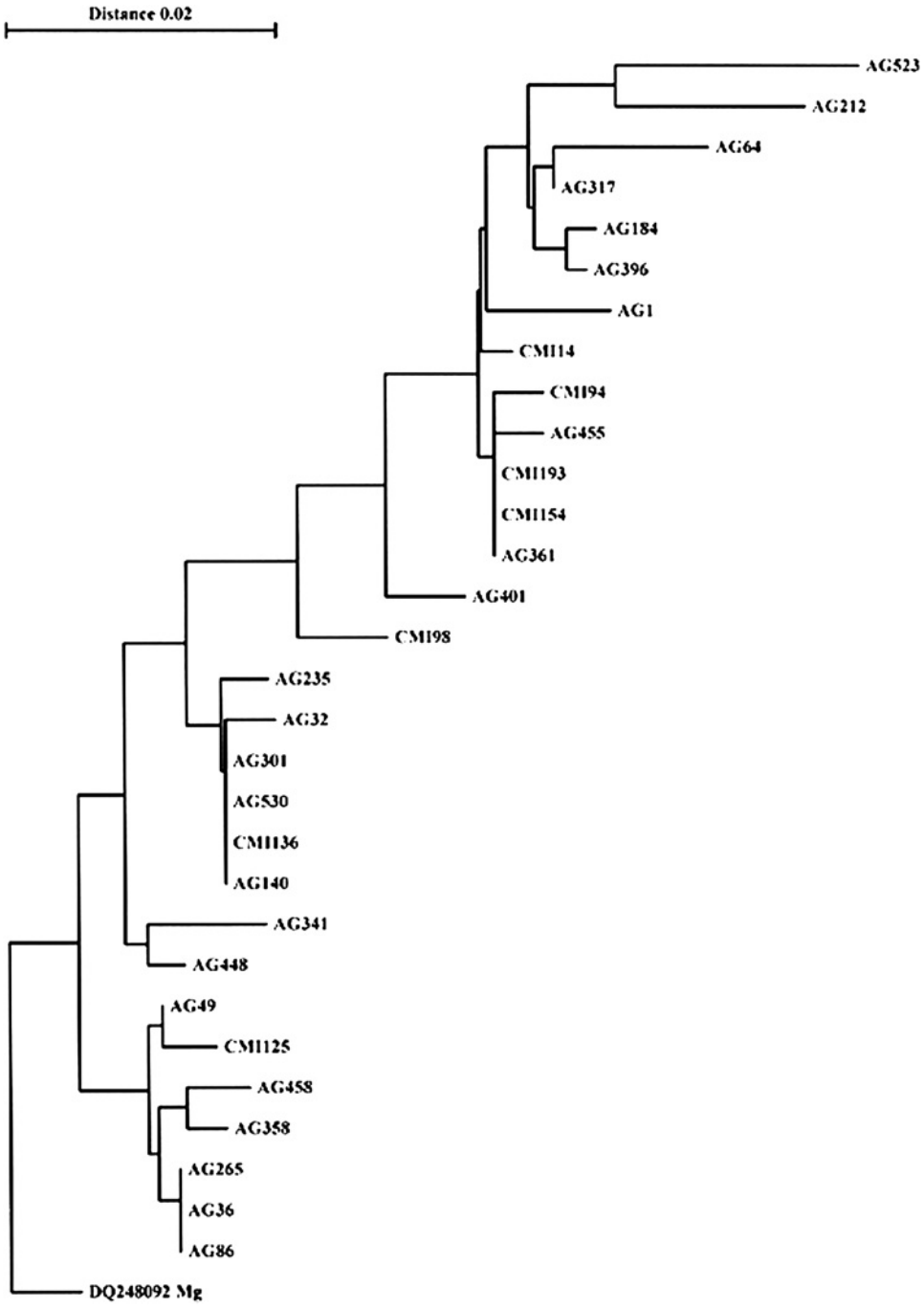

Figure 1 Phylogenetic tree based on a 238 nucleotide sequence of the *Mycoplasma genitalium* MgPa adhesin gene (*mgpB*) in 30 samples from Bissau, Guinea-Bissau. The same sequence from the *M genitalium* reference strain G37^T (DQ248092) was used for rooting the tree.

among women in Guinea-Bissau, has in other settings worldwide been the most frequently detected genovar in infections in men who have sex with men.[12 17–19] This unusual *C trachomatis* genovar distribution among women in Guinea-Bissau may reflect the limited sample size, although it can also indicate a local domestic transmission with little importation of strains from abroad.

The *ompA* sequences were also highly conserved within the genovars. Accordingly, insufficient epidemiological resolution is obtained by *ompA* sequencing, and novel molecular methods for typing of *C trachomatis*, such as multiple loci variable number of tandem repeats (VNTR) analysis (MLVA) and multilocus sequence typing (MLST), which both show a substantially higher discriminatory power, are vital.[31]

Among the 30 *M genitalium* samples, 67% of the *mgpB* STs were only represented by single isolates. Only three small clusters, two containing three isolates and one containing four isolates, were identified. This high genetic diversity may suggest that *M genitalium* is endemic in Bissau and that infection is not due to the distribution of a single strain.

Bacterial STIs are public health problems causing substantial morbidity and economical cost. Furthermore, if undetected and untreated, many bacterial STIs are associated with severe complications and sequelae such as PID, ectopic pregnancy, infertility and facilitated

acquisition and transmission of HIV. Ineffective or inadequate detection and/or treatment of these pathogens inevitably contribute also to further transmission of the STIs. Despite that the present study did not show any alarming tendency of *N gonorrhoeae* strains resistant to extended-spectrum cephalosporins, macrolides and spectinomycin, in Bissau, Guinea-Bissau, the rapidly increasing resistance to also these antibiotics worldwide makes it important to introduce quality-assured gonococcal antibiotic resistance surveillance also in Guinea-Bissau and in general in West Africa. Furthermore, due to the high prevalence of HIV in many of the West African countries and the fact that several of these bacterial STIs also facilitate the acquisition and transmission of HIV, more prevalence studies of bacterial STIs as well as national and regional strategies for prevention and control of bacterial STIs in this region are crucial to implement.

In conclusion, the high diversity of *N gonorrhoeae*, *C trachomatis* and *M genitalium* strains in Bissau, Guinea-Bissau, may be associated with suboptimal diagnostics, contact tracing, case reporting and epidemiological surveillance. Overall, it is exceedingly difficult to estimate the true burden, distribution and characteristics of STIs in Guinea-Bissau, as well as in many other countries in West Africa. Accordingly, additional studies in other population groups, and in other geographical areas, are crucial to perform in Guinea-Bissau and other countries in the region. The present study compiled with such future studies may form the basis for a national sexual health strategy for prevention, diagnosis and surveillance of STIs in Guinea-Bissau.

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STROBE 2007 (v4)Statement—Checklist of items that should be included in reports of *cohort studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	5
Objectives	3	State specific objectives, including any prespecified hypotheses	5-6
Methods			
Study design	4	Present key elements of study design early in the paper	6-7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	6-7
		(*b*)For matched studies, give matching criteria and number of exposed and unexposed	Not applicable
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6-7
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6-7
Bias	9	Describe any efforts to address potential sources of bias	Not applicable
Study size	10	Explain how the study size was arrived at	6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Not applicable
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	Not applicable
		(*b*) Describe any methods used to examine subgroups and interactions	Not applicable
		(*c*) Explain how missing data were addressed	Not applicable
		(*d*) If applicable, explain how loss to follow-up was addressed	Not applicable
		(*e*) Describe any sensitivity analyses	Not applicable
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Not applicable
		(b) Give reasons for non-participation at each stage	Not applicable

		(c) Consider use of a flow diagram	Not applicable
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Not applicable
		(b) Indicate number of participants with missing data for each variable of interest	Not applicable
		(c) Summarise follow-up time (eg, average and total amount)	Not applicable
Outcome data	15*	Report numbers of outcome events or summary measures over time	Not applicable
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Not applicable
		(*b*) Report category boundaries when continuous variables were categorized	8-10
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	Not applicable
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	8-10
Discussion			
Key results	18	Summarise key results with reference to study objectives	11-14
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	11-14
Generalisability	21	Discuss the generalisability (external validity) of the study results	Not applicable
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Not applicable

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.