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Abstract

The emergence *of Neisseria meningitidis* serogroup X (NmX) in the African meningitis belt, urged the development of diagnostic tools and vaccines for this serogroup, especially following the introduction of a conjugate vaccine against meningococci serogroup A (NmA). We have developed and evaluated a new rapid diagnostic test (RDT) for detecting the capsular polysaccharide (cps) antigen of this emerging serogroup. Whole inactivated NmX bacteria were used to immunize rabbits. Following purification by affinity chromatography, the cpsX-specific IgG antibodies, were utilized to develop a NmX-specific immunochromatography dipstick RDT. The test was validated against purified cpsX and meningococcal strains of different serogroups. Its performance was evaluated against PCR on a collection of 369 cerebrospinal fluid (CSF) samples obtained from patients living in countries within the meningitis belt (Cameroon, Côte d'Ivoire and Niger) or in France. The RDT was highly specific for NmX strains. A cut-off of 10^5 CFU/mL and 1 ng/mL was observed for the reference NmX strain and purified cpsX, respectively. Sensitivity and specificity were 100% and 94%, respectively. A high agreement between PCR and RDT (Kappa coefficient of 0.98) was observed. The RDT test gave a high positive likelihood ratio and a low negative likelihood (0.07) indicating almost 100% probability to declare disease or not when the test is positive or negative, respectively. This unique NmX-specific test could be added to the available set RDT tests for the detection of meningococcal meningitis in Africa as a major tool to reinforce epidemiological surveillance after the introduction of the NmA conjugate vaccine.

INTRODUCTION

Neisseria meningitidis (Nm) is an exclusively human capsulated bacterium that can provoke severe invasive infections such as meningitis and septicaemia (1). Meningococcal disease is still a major public health concern due to potential epidemic spread. While the disease occurs sporadically in Europe and North America, it is responsible for major recurrent epidemics within the African meningitis belt (2). The bacterial capsular polysaccharide determines the 12 Nm serogroups currently described. Six serogroups (A, B, C, Y, W and X) are responsible for the vast majority of cases of meningococcal disease worldwide. However, they differ in their global frequencies and geographical distribution (3). This distribution impacts on vaccination strategies, which for the most part involve the established polysaccharide-based vaccines against serogroups A, C, Y and W. Besides, an innovative recombinant protein-based vaccine was recently licensed in Europe and Australia against meningococci of serogroup B (4). This multicomponent vaccine targets conserved proteins among meningococci regardless of their serogroup. Therefore, it has the potential to cover non-serogroup B isolates such as those of serogroup X (5). In the meningitis belt, *N. meningitidis* serogroup A (NmA) predominated prior to the introduction of the NmA polysaccharide-protein conjugate vaccine (MenAfriVac™) (6), while other serogroups (mainly serogroups W (NmW) and X (NmX)), were also detected and still are. Of particular concern, outbreaks due to isolates of NmW and NmX were recently reported in Africa (7-9). Surveillance of the distribution of meningococcal serogroups is therefore important and its comprehensiveness will benefit from diagnosis tools that can be widely used at bedside. We have, in recent years, contributed to the development and validation of immunochromatography dipstick rapid diagnostic tests (RDT) for the identification of *N. meningitidis* serogroups A, C, Y and W (10-11). This major achievement was a first step to improve bedside diagnosis of

meningococcal infection in Niger, a country within the meningitis belt (10,12). While NmX is still rare in Europe (13), its increasing importance in the meningitis belt supports the licensing of an efficient diagnosis device against NmX infection as well as ongoing studies toward a NmX polysaccharide-based vaccine (14). Herein, we report the design, development, and validation in the field of a new RDT for the detection of NmX isolates. Hence, this work contributes to complete the available tools for the diagnosis and surveillance of meningococcal meningitis in the meningitis belt.

Materials and Methods

Bacterial strains and samples

N. meningitidis isolates used in this study were isolates from cases of meningococcal disease (see Table 1 for details). Bacteria were cultured on GCB medium (GC Agar Base, Difco, Detroit, MI, USA) supplemented with Kellogg supplements (15). The serogroup was determined by agglutination with serogroup-specific antisera according to the standard procedure (16). Further phenotyping (serotyping and serosubtyping) was performed using monoclonal antibodies against the meningococcal proteins PorA and PorB as previously described (17). The cerebrospinal fluid (CSF) samples tested in this study corresponded to suspected bacterial meningitis cases. They were obtained from the National Reference Laboratories for Meningococci located at the Institut Pasteur of Côte d'Ivoire and at the Institut Pasteur, Paris, France, as well as from the Centre de Recherche Médicale et Sanitaire (CERMES) in Niamey, Niger, and from the Centre Pasteur of Garoua, Cameroon. These samples were received in the frame of these centres' mission for the surveillance of meningococcal diseases in the corresponding countries under approvals from the internal board of the Institut Pasteur to collect, characterize and use these samples that are all anonymized.

The PCR analysis of these samples was used as a reference method to detect *N. meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*, as well as to genogroup meningococcus-positive specimens. PCR conditions and primers were as previously described (8). Culture was not used as it has been constantly shown to be less sensitive than PCR (18). Culture data were available only for 26 of the 369 tested CSF samples.

Purification of the capsular polysaccharide from NmX

The capsular polysaccharide of serogroup X (cpsX) was purified from the NmX strain 19504 (that gave the highest yield when cultured on GCB medium with Kellogg supplements) by the Cetavlon extraction method as previously described (19). Briefly, bacteria (1 L) at late-logarithmic phase of growth were formaldehyde-inactivated (1% v/v) and then treated with Cetavlon (0.1% w/v) (Sigma Aldrich, France). After centrifugation, the pellet was dissolved in cold aqueous $CaCl_2$ (0.9M). The solubilised materials were cleared by precipitation in 25% aqueous ethanol and the remaining supernatant was precipitated by 80% aqueous ethanol. The pellet was dissolved in phosphate buffer (Na_2HPO_4, NaH_2PO_4, 0.2 M) and treated with Dnase and Rnase followed by proteinase K treatment (Sigma Aldrich, France) and cold phenol extraction. The extract was extensively dialyzed against distilled water and lyophilized to obtain the crude capsular polysaccharide. Ten mg of the preparation were dissolved in 2 mL of phosphate buffer K_2HPO_4, KH_2PO_4 (0.05 M), pH 7, and purified by gel filtration on a Biosep-SEC-S3000 column (300 x 21.2 cm, Phenomenex, France) that was equilibrated with the same buffer. Elution was carried out with the same phosphate buffer at 5 mL/min, and monitored at 214 nm and 280 nm. The void volume fractions containing cpsX in the high molecular-weight range were pooled and dialyzed against distilled water at 4°C, using a dialysis membrane with a cut-off size of 10K-15K, and the residue was lyophilized. The yield was about 20 mg/L of culture. The profile of the purified cpsX was checked by proton nuclear magnetic resonance (^{1}H NMR) (data not shown) as previously described (20). CpsA, cpsB, cpsC, cpsY and cpsW were similarly purified from five strains of serogroups A, B, C, Y and W (strains 21524, 21721, 22639, 16366 and 19995 respectively, Table 1).

Rabbit immunization and purification of specific anti-cpsX IgG antibodies

Two New Zealand White female rabbits (3 kg) were immunised intravenously three times with doses of 1mL of a suspension of 10^9 colony forming units, CFU, of freshly heat-inactivated

NmX strain 19504 (30 min at 56°C), at day 0, 7 and 21. Sera were taken before immunization and at day 28 after the fist injection to evaluate the immune response by ELISA (see below). Dot blotting with rabbit sera (1:1000 serum dilution) was performed using Amersham ECL kits (GE Healthcare Life Sciences Velizy-Villacoublay, France) as previously described (21). Rabbit immunisation was performed according to the European Union Directive 2010/63/EU (and its revision 86/609/EEC) on the protection of animals used for scientific purposes. Our laboratory has the administrative authorization for animal experimentation (Permit Number 75–1554) and the protocol was approved by the Institut Pasteur Review Board that is part of in the Regional Committee of Ethics of Animal Experiments of the Paris region (CETEA 2013-0190).

IgG antibody purification was performed by affinity chromatography in two steps. First, the rabbit's sera were passed through a HiTrap Protein G HP column (GE Healthcare, France) and eluted with glycine-HCl 0.1 M pH 2.7. Fractions of 1 mL were recovered in 50 µL of Tris-HCl buffer (1 M, pH 9). Fractions were tested for protein content by measuring their absorbance at 280 nm. Pooled fractions were passed through a cpsX affinity column obtained by chemical coupling of the amine functions of the CarboxyLink resin and the phosphate functions from cpsX, according to manufacturer recommendations (Thermo Scientific, Rockford, IL. USA). The eluted fractions were tested by ELISA against purified cpsX and whole inactivated NmX bacteria. To do so, ELISA wells were coated overnight with 100 µL of a solution containing 2 µg/mL of purified cpsX or 100 µL of a bacterial suspension of $3x10^8$ CFU/mL (NmX strain 19504). The purified antibodies (at a 500 pg/ml concentration) were tested against serial dilutions of bacteria from serogroup A, B, C, Y, W and X in a dot blot experiment, and serial dilutions of the antibodies were then tested in ELISA on counterpart coated cps at 2 µg/mL concentration.

Production and validation of a RDT against NmX

A one-step vertical flow immune-chromatography dipstick was set up using purified cpsX-pAbs that were conjugated to gold particles (British Biocell International, Cardiff, UK) as previously described (22). Unconjugated cpsX-pAbs were used as capture antibodies and goat anti-rabbit IgG (ICN Biomedicals, Aurora, Ohio, United States) were used as control antibodies. Both types of antibodies were sprayed onto nitrocellulose (Schleicher & Schuell Bioscience, Ecquevilly, France) at 2 µg and 1 µg per line centimeter respectively. For the test evaluation, dipsticks were dipped, for a 10-15 min period at room temperature, in 100 µL of PBS containing bacterial suspensions or CSF samples.

Data analysis

Sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) were calculated using a 2 x 2 contingency table**.** The positive likelihood ratios LR (LR^+ = Se/[1-Sp]) and the negative LR (LR^- = [1-Se]/Sp), were also calculated (23). These values give an indication of the likelihood that the sample is positive or negative prior to testing. The diagnostic odds ratio (DOR), defined as the ratio of the odds of positive test results in specimens with NmX on the odds of positive test results in specimens negative for NmX, was calculated as follows DOR = (Se/[1 – Se])/([1 –Sp/Sp] (24). Finally, the Cohen's kappa (j) statistic was calculated to measure concordance between PCR and RDT (25).

RESULTS

Characterization of rabbit anti-meningococcal serogroup X rabbit serum

Following the three dose-immunization regimen with whole NmX bacteria, the rabbit sera were tested in dot blot analysis against spotted bacteria. While no bacteria detection was obtained with control pre-immune sera, a strong detection was obtained with the sera from immunized rabbits (Fig.1). Sera from the two responding rabbits were pooled and anti-cpsX-specific IgG were purified by affinity chromatography on a NmX cps activated column. Dot plot analysis of the purified IgG response against decreasing numbers of bacteria (from $5x10^5$ to $5x10^3$ cells per spot) from serogroups A, B, C, Y, W and X showed that antibodies only recognized serogroup X strain (Fig. 2A). The absence of recognition of the other serogroups (A, B, C, Y and W) was further confirmed independently by ELISA analysis of the antibody response against coated (1 µg/mL) purified cps corresponding to the six serogroups (Fig. 2B).

A dipstick rapid diagnostic test for NmX was produced (see Material and Methods), and its detection limits were established. For the purified cpsX, this limit was 1 ng/mL (Fig. 2C) and was 10^5 CFU/mL (data not shown) for NmX bacteria (strain 19504) The cut-off analysis was repeated 3 times with identical findings that were not affected by dipstick storage for 3 weeks at 25°C. We also tested the RDT on a collection of bacterial suspension (Table 1) at 10^6 CFU/mL. Only the serogroup X isolates were detectable (data not shown). As different concentrations of antibodies were used in these assays, the data suggest that the concentrations of antibodies do not preclude detectable reactivity with other serogroups.

Use of the NmX dipsticks on clinical samples

The NmX dipstick was tested on a panel of 369 CSF selected from historical collections kept in National Reference Centre/Laboratory from four different countries, differing in terms of

meningitis incidence (Cameroon, Côte d'Ivoire, France and Niger). Noticeably, three out of the four laboratories are located in countries within the meningitis belt. The CSF samples corresponded to suspected cases of acute bacterial meningitis. They were characterized by PCR for etiological diagnosis (Table 2). Culture results were only available for 26 samples (8 samples positive for *S. pneumoniae*, 4 positive for *N. meningitidis* (2 serogroup B and 2 serogroup W), 1 positive for *H. influenzae*, 1 positive for S. *agalactiae* and 12 CSF samples were sterile by culture).

Among these isolates, 52% (n = 191) were positive for Nm, 8% (n = 28) were positive for other bacterial species, namely *S. pneumoniae*, *H. influenzae* and *S. agalactiae*, and 40% (n = 150) were negative by PCR for these species. Among the Nm positive CSF, the six meningococcal capsular groups involved in invasive meningococcal infections were represented: group A (n = 27), group B (n = 8), group C (n = 7), group Y (n = 2), group W (n = 38) and group X (n = 92). In addition, 17 CSF samples were positive for Nm by PCR although they were negative for groups A, B, C, Y, W and X. All samples that were negative for NmX by PCR were also negative for this group by the new NmX-specific RDT. Among the 92 CSF positive for NmX by PCR, 86 were also positive by RDT. All the 26 CSF samples with culture data were tested negative by NmX-specific RDT (data not shown).

We also conducted a limited prospective analysis during the epidemic season 2014 in the three laboratories located in countries of the meningitis belt. We tested all the 153 CSF samples that were received in the three laboratories in Cameroon, Côte d'Ivoire and Niger between 1 January 2014 and 15 March 2014. No NmX was detected by PCR or by RDT in any of the samples. In contrast, several samples were positive by PCR for *S. pneumoniae* (14%), NmW (7%) and *H. influenza*e (3%).

Performance of the NmX-specific RDT: sensitivity, specificity, likelihood ratios, and predictive values

RDT data showed a good correlation with PCR data, indicating a Kappa correlation coefficient of 98%. The sensitivity, specificity and 95% CI (confident interval) data of the RDT obtained for the documented 369 CSF samples are summarized in Table 3. The specificity of RDT for CSF infected by NmX was 100%, while the sensitivity reached 94%. Calculating the positive likelihood LR^+ and DOR was not feasible due to a Sp value of 100%. LR^+ and DOR values were therefore calculated using a value for the specificity that corresponded to the lower 95% confidence interval for specificity (0.99) (Table 3).

The prevalence of NmX among the 369 tested CSF was 25%. Therefore, the NPV and PPV are given in Table 3 under this prevalence value. However, the tested samples were selected from the collections of the participating laboratories and may not reflect the real prevalence of the disease. Moreover, the frequency of NmX meningitis may also vary across time and countries within the meningitis belt and elsewhere. We therefore calculated the negative and positive predictive values (NPV and PPV) according to a prevalence varying from 0 to 100%, using the Se and Sp obtained from the CSF samples in this study (Fig. 3).

DISCUSSION

Reliable tests for the identification of cases of meningococcal meningitis and serogroup-determination are crucial to ensure proper individual (case-by-case) as well as collective management of cases and epidemiological surveillance. Culturing *N. meningitidis* may frequently fail due to early antibiotic treatment and fragility of this bacterial species (26). During the last two decades, PCR-based nonculture methods have been developed, enabling a significant improvement of the management and surveillance of bacterial meningitis (18). PCR-based methods require specific laboratory equipment and trained staff and can not be used as a bedside method. Nevertheless, the PCR technology was implemented in several reference laboratories located in countries within the African meningitis belt (18). However, PCR may not be sufficiently set to ensure country-wide surveillance, especially in populations leaving in remote areas. Other tests, such as the currently available latex agglutination kits, require trained staff and an unbroken cold chain for storage and distribution of the kits.

The recent implementation of RDT for meningococci of serogroups A, C, Y and W was a major breakthrough for individual diagnosis and for surveillance of meningococcal diseases in the African meningitis belt (12). These tests are stable at temperature up to 45°C at least. They are easy to use and to interpret in the absence of extensive training, and therefore are adapted for bedside use. The emergence of meningococcal isolates of serogroup X urged the development of a RDT test for this serogroup to complete the current RDT tools. We first analyzed the inherent quality of such a serogroup X specific test. The specificity and sensitivity parameters were evaluated under laboratory conditions using a selected panel of relevant CSF samples. The good quality of the new RDT was reflected by its high sensitivity and specificity for NmX with a very high likelihood ratio for positive test (Table 3).

We also evaluated its usefulness that depends not only on the quality of the test but also on the prevalence of the NmX meningitis in the tested population. The prevalence of NmX within the panel of CSF samples that was used to evaluate the RDT specificity and sensitivity was 25.7%. It may not properly reflect the real prevalence of NmX in areas at risk. Usefulness is usually evaluated using two parameters, the PPV and NPV. When NmX prevalence was forced to vary between 0 and 100%, the PPV remained stable at 1 indicating that the test remained highly proficient in ruling-in a case. Moreover, the NPV retained high values when the prevalence of NmX was very low. In addition, the test remained proficient (NPV of 0.95 or higher) if this prevalence increased to 50%. These considerations seem realistic and reflect the current epidemiological situation in the meningitis belt after the introduction of MenAfriVac™ that was associated with significant decrease of NmA (9). Indeed, this small scale prospective use of the new RDT in the three centres located in this area (Abidjan, Garoua and Niamey), suggests, on the basis of sensitivities of RDT and PCR (that are less than 100%), that NmX may be present albeit not as a dominating pathogen. In contrast, NmW was the most frequently isolated Nm species, while most cases were associated to *S. pneumoniae*. However, a large-scale multi-site prospective study comparing PCR and all the available RDT (A, C, Y, W, Y and X) is warranted in the future. Moreover, additional work is required to miniaturize the RDT to be applicable on small volume samples (< 100 µL).

In summary, this work reports a new reliable and rapid diagnostic test to detect serogroup X that should enhance diagnosis of meningitis due to this serogroup and is expected to improve epidemiological surveillance. Epidemiological changes upon the implementation of MenAfriVac™ can be better monitored. This test would be helpful for the development and the implementation of vaccines with broad serogroup coverage that can target NmX (5) or with NmX-specific vaccines (14).

LEGENDS OF FIGURES

Figure 1. Dot blotting analysis of rabbit sera. Sera from two rabbits prior to immunization (day 0) and 7 days after injection of the third dose of NmX strain 19504 (day 28) were used at 1:1000 dilutions in immunoblotting. Four meningococcal isolates were spotted at 2.10^5 colony forming units, CFU/mL (1: strain 19404, 2: strain 23557, 3: strain 24196, 4: strain 24287).

Figure 2. Specific recognition of the purified rabbit anti-cpsX IgG antibodies. (A) Dot blotting analysis against whole bacteria. Serogroups are indicated above the dots and amounts of loaded bacteria in each spot are indicated on the right (in colony forming units, CFU). Antibodies were used at a final concentration of 500 pg/mL. (B) ELISA analysis using coated purified capsular polysaccharide for serogroups A, B, C, Y, W and X (Table 1). Data are expressed as OD 492 nm absorption for each concentration of antibodies (in pg/mL). Data correspond to the means of two independent experiments. The corresponding serogroups are indicated on the right. (C) Detection cut-off value for purified cpsX. The amounts are indicated in ng above each dipstick. A dipstick, before use, is shown on the left. The upper two arrows indicate the capture control line corresponding to the goat anti-rabbit IgG. The lower two arrows indicate the capture line corresponding to the anti-cpsX-specific IgG (cpsX line).

Figure 3. Predictive values for *N. meningitidis* diagnosis. Positive Predictive Values and Negative Predictive Values (PPV and NPV, respectively) for the diagnosis of NmX were calculated according to a disease prevalence ranging between 0 and 100%.

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Table 1. Strains used in the study and their characteristics

Strain reference	Serogroup:serotype/serosub-type
21525*	A:4:P1.9
21526	A:4:P1.9
19256	B:NT:P1.5,2
19257	B:2a:P1.5,2
19324	B:2b:P1.5,2
21721*	B:NT:P1.4
22733	B:15:P1.4
22590	B:14:P1.7,16
22644	C:15:P1.7,16
22639*	C:2a:P1.5
20137	C:2b: P1.5,2
19008	C:2a: P1.5,2
20134	C:NT:P1.10
19456	Y:14:NST
19336*	Y:NT:P1.5
19995*	W:2a:P1.5,2
19481	W:NT:P1.5
19836	W:NT:P1.6
19383	E:NT: P1.5,2
19504*	X:NT: P1.5,2
24196	X:4:P1.12
24287	X:4:P1.16
23557	X:NT:P1.5

NT: Nontypeable; NST: Nonsubtypeable.

* Strains that were used for capsular polysaccharide purification

Table 2 Results of CSF samples obtained by PCR and by RDT

	Geographical origins					RDT	
PCR	IP Paris	CERMES	CP Garoua	IP Côte d'Ivoire	Total	NmX^+	NmX^-
NmA	6	15	6	0	27	0	27
NmB	6	0	0	2	8	0	8
NmC	7	0	0	0	7	0	7
NmY	2	0	0	0	2	0	2
NmW	6	10	4	18	38	0	38
NmX	7	80	5	0	92	86	6
Nm NG	0	0	16	1	17	0	17
S. pneumoniae	0	0	10	13	23	0	23
H. influenzae	0	0	1	3	4	0	4
S. agalactiae	1	0	0	0	1	0	1
Negative*	10	0	77	63	150	0	150
Total	45	105	119	100	369	86	283

*PCR Negative for *N. meningitidis*, *S. pneumoniae* and *H. influenzae*

CSF; Cerebrospinal fluid, RDT: Rapid Diagnostic Test, IP= Institut Pasteur, CP: Centre Pasteur, Nm: *Neisseria meningitidis*, NG non groupeable

Table 3 Performance of the RDT for NmX

Test parameter	Value	95% confidence interval
Sensitivity (Se)	0.94	0.86 to 0.98
Specificity (Sp)	1	0.99 to 1
Positive Likelihood ratio (LH^+)*	94	32 to 8252
Negative Likelihood ratio (LH^-)	0.07	0.03 to 0.15
Positive predictive value (PPV)	1	0.96 to 1
Negative predictive value (NPV)	0.98	0.95 to 0.99
Diagnostic odd ratio (DOR)*	1567	379 to 118420

Dividing by zero; the values of LH^+ and DOR were calculated using a value for specificity that corresponded to the lower 95% confidence interval (0.99).

Fig. 1

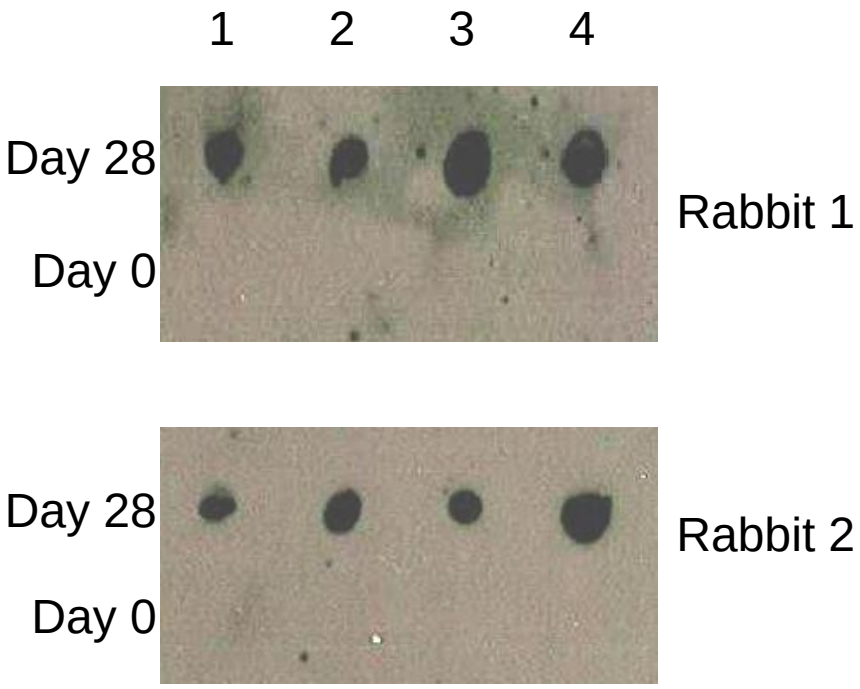
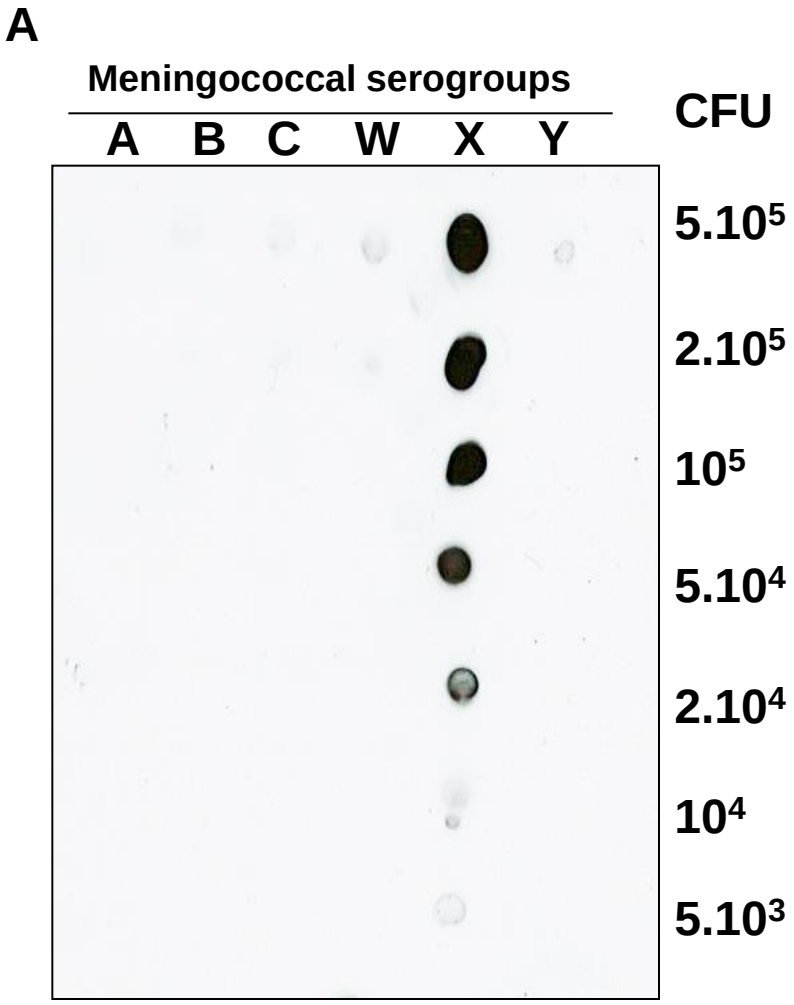
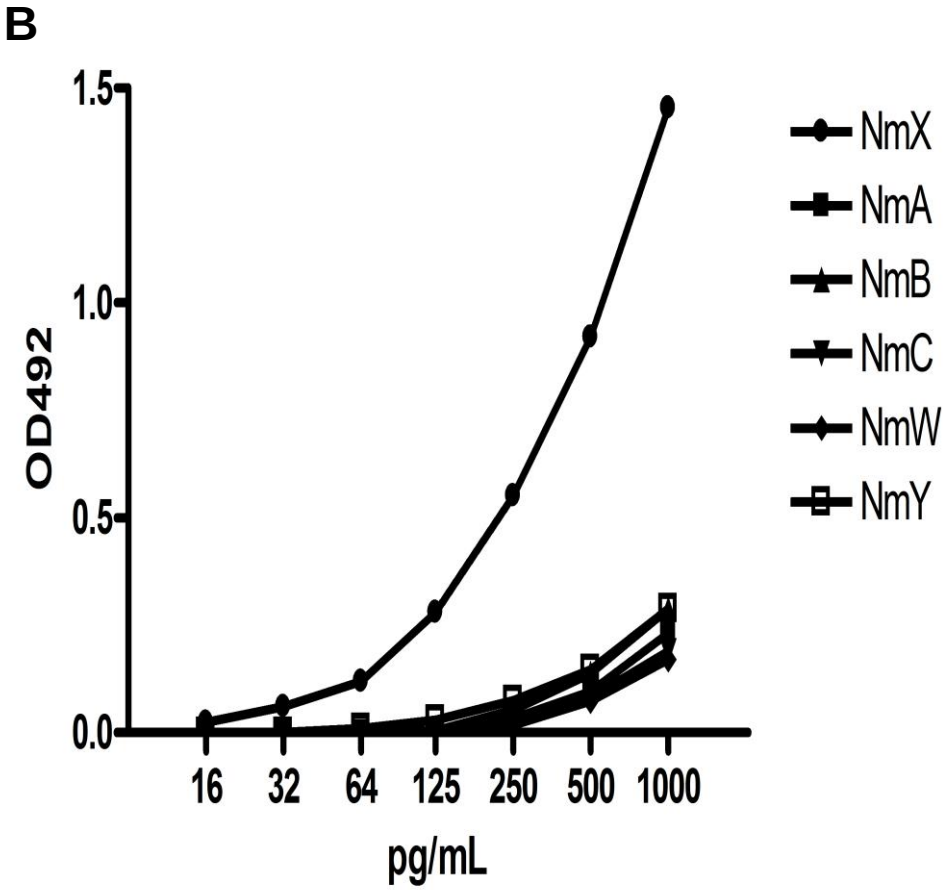


Fig. 2


A
Meningococcal serogroups
A B C W X Y
CFU
5.10^5
2.10^5
10^5
5.10^4
2.10^4
10^4
5.10^3


B
OD492
1.5
1.0
0.5
0.0
16 32 64 125 250 500 1000
pg/mL
NmX
NmA
NmB
NmC
NmW
NmY

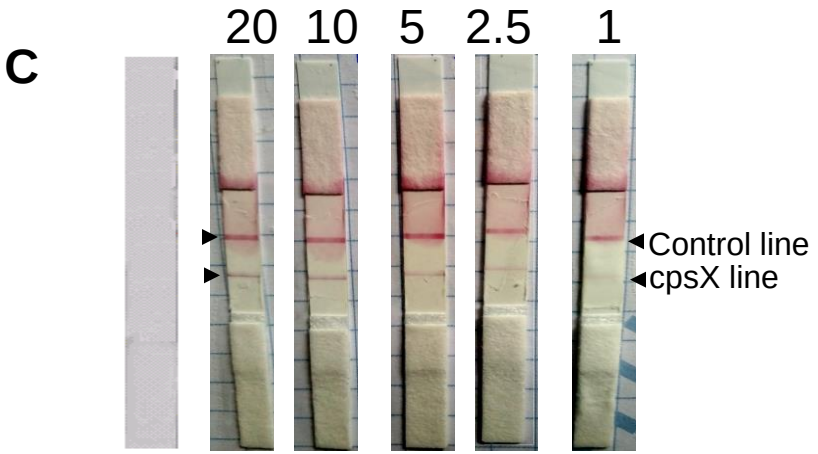
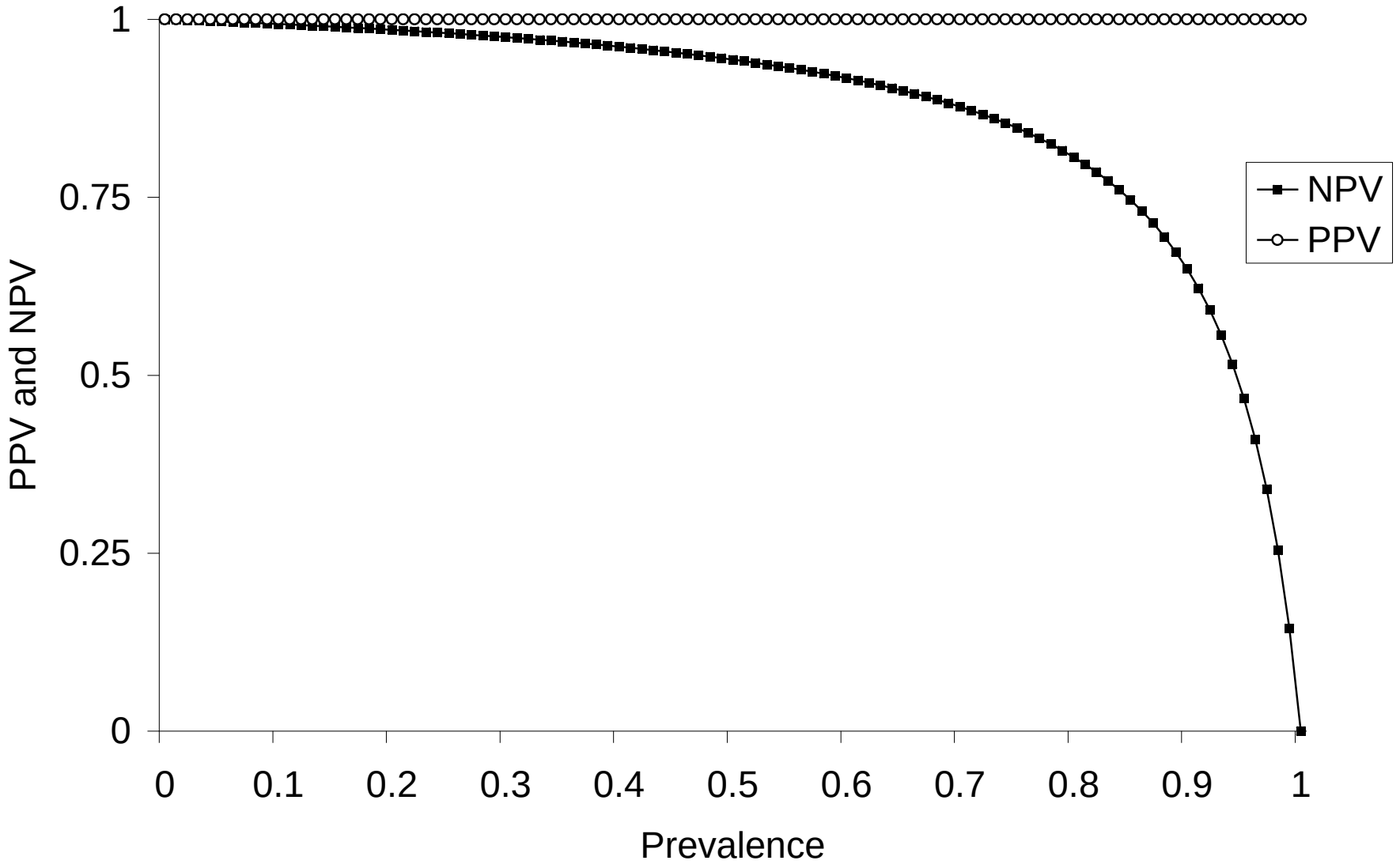

C
20 10 5 2.5 1
Control line
cpsX line

Fig. 3


1
0.75
0.5
0.25
0
PPV and NPV
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Prevalence
NPV
PPV