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FOREWORD

In South Africa the public sector HIV care and treatment programme is delivered through a mixture of health care facilities which collectively test an estimated 10 million people per year. The provision of HIV testing, linkage and retention in HIV care and treatment with antiretroviral therapy has proven remarkably successful yet males are lagging in terms of programme outcomes. The reasons for this disparity are reviewed in this issue. Also under review is the incidence of non-typhoidal *Salmonella* in imported and locally produced poultry and other foodstuffs in relation to the African Growth and Opportunity Act.

Assessments of the efficacy of transmission blocking vaccines for malaria control require complex *Plasmodium* and *Anopheles* culturing and infection systems. The Wits Research Institute for Malaria (WRIM) has recently established a host-parasite infection facility and has succeeded in artificially infecting several *Anopheles* laboratory strains with a *Plasmodium falciparum* gametocyte culture. The details of these infections by *Anopheles* species are given in this issue. Of special note, these data represent the first successful laboratory infections of *An. funestus* and *An. arabiensis* with *P. falciparum.* Lastly, a variety of different preparative protocols were recently assessed by the Electron Microscope Laboratory of the NICD, for staining the capsules of Gram-positive and Gram-negative bacteria, with the ultimate aim of describing the ultrastructure of selected non-typeable bacterial isolates identified during national laboratory-based surveillance for invasive bacterial respiratory pathogens. The results of these protocol assessments are given in this issue.

All contributors are thanked for their inputs, and we trust you will find these reports useful and interesting.

Basil Brooke, Editor

CONTENTS

GENDER DISPARITIES IN HIV CARE AND TREATMENT IN SOUTH AFRICA: A REVIEW OF LOCAL LITERATURE

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Introduction

South Africa has made tremendous progress in its response to the HIV epidemic. This includes the provision of HIV testing, linkage and retention in HIV care and treatment with antiretroviral therapy. The public sector HIV care and treatment programme is delivered through a mixture of health care facilities including primary care facilities and has grown from a few such facilities in 2004 to more than 3800 in 2015.[1] By the end of 2015, the programme was testing an estimated 10 million people per year and there were 3.3 million people remaining on antiretroviral therapy (ART).[1,2]

There have been concerns from many quarters that while there is good progress overall, males are lagging in terms of programme outcomes such as ART initiation, retention in care and mortality on or off ART. Although recent systematic reviews of global literature did not find significant differences between males and females with respect to CD4 count recovery and virological suppression or failure, they did find increased risk of mortality and progression to AIDS amongst males in low and middle income countries.[3,4] Currently, South Africa faces a quadruple burden of diseases including communicable diseases such as tuberculosis (TB) and HIV, non-communicable diseases, perinatal and maternal diseases as well as injuries. The country also has high levels of alcohol and substance abuse which are more prevalent in males. All these factors may contribute to adverse outcomes amongst males receiving HIV care and treatment.

Against this backdrop a review of South African literature was conducted to determine the extent of gender disparities in HIV care and treatment and to identify factors associated with it. It is envisaged that the findings of this review will inform the design and implementation of interventions or surveillance activities within the national HIV care and treatment programme.

Methods

Search strategy

A keyword search in Google scholar was conducted using the following search terms “HIV testing”, “linkage”, “retention”, “CD4 count”, “viral suppression”, “mortality”, “gender” and “South Africa”. Google scholar was selected for convenience and also to improve yields from sources other than peer reviewed journals indexed in PubMed. The search was limited to publications from 1st January 2010 until 30th April 2016 and published in English. From the initial titles retrieved, a title screen was conducted in order to identify publications for abstract review. From the abstract review, publications for full text review were identified. For abstracts to be eligible for full text review, they had to report data on cohorts from which any of the following outcomes were measured: linkage to care, ART initiation of first line ART, CD4 count recovery or gain, retention in care, viral suppression, or mortality in adults aged 15 years or older by gender. If the outcomes were not reported by gender, there had to be evidence that the authors conducted adjusted analyses including gender which would permit extraction of data on relevant outcomes by

gender. If studies were multi-country, there had to be evidence that data from South Africa was reported separately.

Data extraction and analysis

From the full texts reviewed, studies which met the eligibility criteria for inclusion in the study were identified. From eligible studies, data on the following study characteristics were extracted: year of publication, location, description of study cohort (population, size, proportion of males, median age), outcomes measured and their definitions, study findings according to the outcomes and any other factors independently associated with the outcomes. If one paper described more than one eligible cohort, data on all eligible cohorts were extracted. Data were extracted and entered into an Excel [Microsoft ®, Seattle, Washington, USA] spreadsheet. Data from the spreadsheet were then exported into Stata® 14 [Stata Corporation, College Station, Texas] for analysis. Descriptive statistics were used to describe the studies according to study characteristics and outcomes. The study findings were summarised qualitatively and associated factors were described.

Results

From an initial 1540 titles retrieved, the first unique 601 titles (39%) were screened. From these titles, 120 abstracts were reviewed and 53 abstracts were identified for full text review. Following full text review of the 53 papers, 20 papers describing 23 cohorts were found to be eligible for inclusion in the review. The most common reasons for exclusion of papers at the full text review were outcomes not being reported by gender [14 (43.7%)] and studies reporting irrelevant outcomes [10 (34.4%)].

Description of studies

Of the 20 studies included in the review, more than half were published prior to 2013. Most studies reported data collected in three provinces – Gauteng, KwaZulu-Natal and Western Cape with only three studies including other provinces. The median proportion of males in the cohorts was 35% (range 11.1- 54%), the median of median ages reported was 35 years (range 31- 38 years) while the median of the median CD4 counts at ART initiation was 101 cells/µl (range 81- 132 cells/µl). Median duration of follow up was reported by 11 of the 20 studies and ranged from 0.8 – 3 years. The most common outcomes reported in the studies were mortality (n=12), retention (loss to follow up or default, n=7) and CD4 gains/ response to ART (n=5). For source information and summary details of each study, please contact Dr Tendesayi Kufa-Chakezha at TendesayiKC@nicd.ac.za.

Summary of findings

1. Mortality

The mortality rates reported for women ranged between 3.4 - 11.8 per 100 person-years while that for males ranged between 5.1 – 20.3 per 100 person-years. For eight of the 12 studies in which gender was independently associated with mortality, males were 11-40% more likely to die during ART. Other factors which were independently associated with increased mortality were CD4 counts < 100 cells/µl at start of ART, current CD4 counts <100 cells/µl, late WHO stage at ART start, anaemia, low BMI and lack of virological suppression. Two studies showed that although mortality decreased with increasing duration on ART, the gender disparity in mortality increased with increasing duration on ART. One study examined the ratios of female: male mortality by age amongst HIV-positive individuals on ART and compared them to the same ratios amongst HIV negative individuals and found that the gender disparities in mortality were less among the HIV positive individuals compared to the HIV negative individuals, suggesting that higher non-HIV mortality in males on

ART compared to females on ART may also contribute to this gender disparity in mortality.

2. Loss to follow-up or default

The proportion of females who were lost to follow-up or defaulted from ART using study specific definitions ranged from 9.8% - 21.6% compared to 11.8- 27.9% amongst males. In five of the seven studies in which gender was independently associated with loss to follow -up or default, males were 18- 51% more likely to be lost to follow-up or to default from ART compared to females. Other factors associated with increased default or loss to follow up were lower CD4 counts at ART start (although one study found an association of loss to follow up with higher CD4 counts), younger age and increasing calendar years.

3. Viral suppression or virological failure

Three studies reported on virological suppression or virological failure. From these studies males were 20-36% less likely to be virally suppressed but were no more likely than females to develop virological failure. Other factors independently associated with lack of viral suppression were younger age, lower CD4 counts and use of nevirapine based regimens.

4. CD4 count gains

In four of five studies which reported on CD4 count gains, females were more likely to have statistically significant higher CD4 count increases after adjusting for other covariates. The fifth study reported no differences in the proportions of males and females with CD4 counts < 200 cells/µl at the start of ART who still had CD4 counts < 200cells/µl after 12 months in spite of being virally suppressed.

5. Linkage to care and ART initiation

Three studies reported on linkage to care and ART initiation in four cohorts and all showed trends towards females being more likely to initiate ART in the defined time periods compared to males, although the differences were not statistically significant in two cohorts.

Discussion and conclusions

The review of studies from South Africa revealed that males in HIV care and treatment are more likely to start ART late, are less likely to be virally suppressed or retained in care and are more likely to die in the follow-up period after ART initiation after adjusting for pre-treatment factors and, in a few studies, most recent CD4 counts and viral loads. These studies also reported on other factors independently associated with adverse outcomes including low CD4 counts, lack of viral suppression and duration on treatment. Most of the studies did not provide reasons for these gender disparities but discussed possible biological and socio-behavioural factors which could not be adequately adjusted or controlled for in the analyses.

It is likely that there are multiple factors which may account for gender disparities in HIV care and treatment. One factor may be the age at which males acquired HIV infection and subsequently started ART. From the 2012 Human Sciences Research Council (HSRC) survey, HIV incidence peaked in the 15- 24 year age group amongst females compared to the 25-49 year age group amongst males.[6] This age gap in HIV acquisition likely translates into an even wider age gap at the initiation of ART, taking into account late ART initiation by males.[26] Older age at ART initiation is independently associated with mortality and sub-optimal response to ART in the presence of viral suppression.[5,8,9] Other factors suggested in recent reviews and studies include: biological differences in innate immunity; differences in dietary practices with males more likely to experience micronutrient deficiencies linked to the immune response to HIV;

higher likelihood of males experiencing internalised stigma within the health system and therefore less likely to seek care timeously; higher likelihood that younger, single and unemployed males struggle to initiate ART and remain on treatment.[7,10,11]

This review was limited to data from South African cohorts in order to maximise the generalizability of findings to the country's HIV positive populations. However, the review had some limitations. Firstly, this was not an exhaustive systematic review and may have missed studies reporting contrary findings. Studies identified in this limited review included most of the large treatment cohorts in the country and it is unlikely that any missed studies would have found results that are very different from those reported here. Another limitation was that the studies used different definitions of the outcomes. Because the review was not attempting to calculate summary estimates for the outcomes but rather summarise the effect sizes and their directions, the studies were still useful despite this limitation. A further limitation was that the studies had limited durations of follow-up ranging from just under one year to three years. Findings on outcomes such as mortality and virological suppression or failure may look different at longer durations of follow-up.

The findings from this semi-systematic review suggest that more interventions to improve the early uptake of HIV testing, ART initiation, adherence to treatment and retention in care amongst males need to be evaluated. These interventions may include extending clinic opening hours to after-hours or weekends to cater for working populations, decongesting clinics to reduce clinic waiting times and use of peer support. [12,13] From a surveillance perspective, these gender disparities show that it is imperative to record and report gender-specific data and to develop gender-specific HIV care cascades in evaluating progress towards the 90-90-90 targets. Further research into the modifiable factors which account for these gender disparities are also needed and the contribution of non-HIV causes to morbidity and mortality among people on ART is also needed.

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NON-TYPHOIDAL *SALMONELLA* AND THE AFRICAN GROWTH AND OPPORTUNITY ACT

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The WHO has reported a massive burden of foodborne disease internationally with 600 million incidences of food-borne illnesses corresponding to 400 000 food-borne deaths annually. It is also reported that 40% of illnesses occur among children <5 years of age.[1] These estimates are believed to be conservative and do not take into account additional challenges such as antimicrobial resistance. Annually, non-typhoidal *Salmonella* (NTS) account for ~80 million food-borne illnesses and 30,000 deaths internationally.[1]

The African Growth and Opportunity Act (AGOA) is a United States (US) Trade Act, enacted in 2000 and recently renewed. It enhances market duty-free access to the US for qualifying sub-Saharan African (SSA) countries. It includes bilateral trade between countries, and thus includes US food imports into South Africa. Prior to the renewal of this act there were concerns regarding job losses in South Africa as well as possible contamination of meat carcasses by pathogens. Until 2015, imports of chicken from the US were negligible for the South African market. It is important to note however that stringent guidelines exist in the US for chickens destined for slaughter (Table 1), and there is zero tolerance for the occurrence of enteric pathogens, including *Salmonella*, in pre-prepared foods.

Table 1: Indicator Organism Median Values for Chickens Median (CFU/mL) following US Department of Agriculture guidelines.[2]

	Generic *E. coli*	APC	Enterobacteriaceae	Total Coliform
Carcass – *Rehang*	540	28,000	1,600	940
Carcass – *Post Chill*	20	260	20	20

National surveillance through the GERMS-SA programmes has highlighted that the commonest NTS in South Africa are *Salmonella enterica* serotype Typhimurium (*Salmonella* Typhimurium) and *Salmonella* Enteritidis. There is strong evidence to suggest that both these pathogens may predominantly be associated with human-to-human spread in Africa.[3,4] Nevertheless, there is a clear record of food-borne outbreaks in South Africa associated with certain *Salmonella* spp., including *Salmonella* Enteritidis. Laboratory-based surveillance for NTS has revealed that a number of these pathogens have caused outbreaks, and both chicken- and beef-associated isolates have been described in the affected human populations (Figure 1).[5,6]

Despite the absence of systematic laboratory-based surveillance in 2014 and 2015, *Salmonella* Saintpaul, which was previously associated with extensive food-borne outbreaks in the US,[6] appears to be increasing and a peak in 2009 of *Salmonella* Infantis is probably related to an unpublished outbreak in that year (Figure 1).

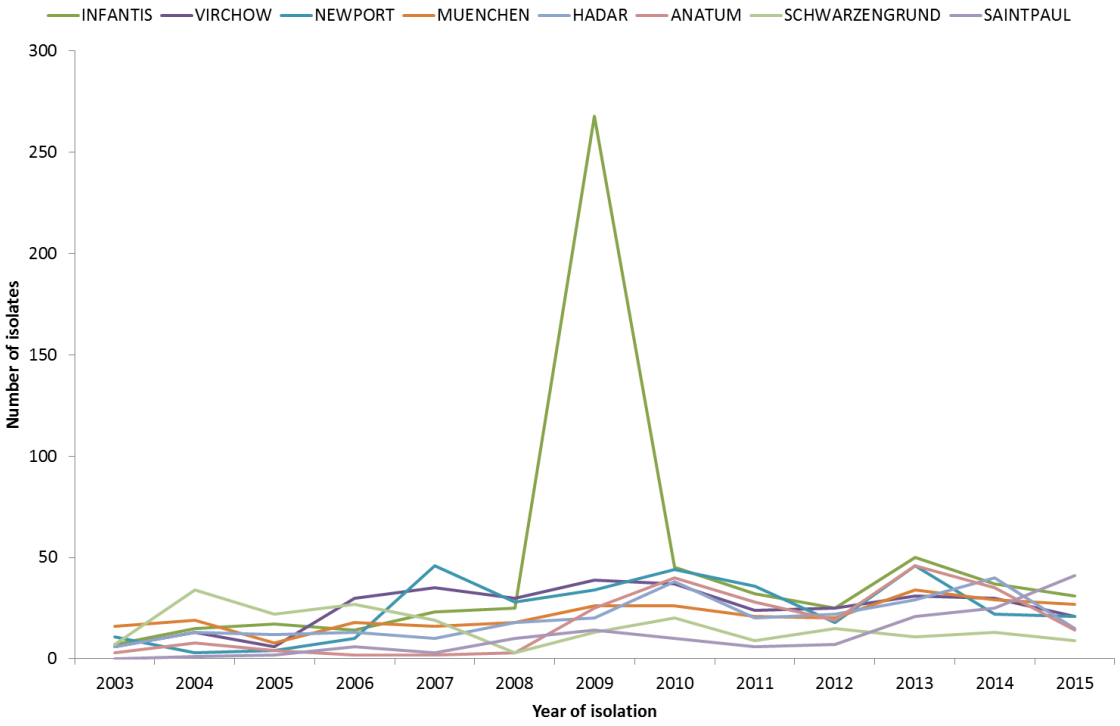

Figure 1: Selected *Salmonella* serotypes, excluding potential human-to-human transmission of *Salmonella* Typhimurium[3] and *Salmonella* Enteritidis[4], known to be associated with foodborne outbreaks internationally, isolated from South African patients, 2003 – 2015. Data for 2014 and 2015 are incomplete due to the absence of formal laboratory-based surveillance.

Whilst data on the incidence of *Salmonella* serovars in animals in South Africa are available, knowledge of the true burden of disease in the human population is limited. Regulations are in place to control *Salmonella* spp. in food animals, primarily targeting *Salmonella* Enteritidis in poultry, [Section 31 of the Animal Diseases Act (Act 35 of 1984)]. The Meat Safety Act (2000) also requires preventive strategies and hygiene management to reduce, eliminate or prevent potential foodborne hazards. In South Africa, *Salmonella* spp. are defined for the microbiological monitoring of imported meat, due to the association with serious illness or death, including antimicrobial-resistant isolates.[8]

Laboratory-based surveillance has additionally revealed that common serotypes associated with NTS infection in the US do occur in South Africa (Table 1). In the US, the vast majority of outbreaks caused by *Salmonella* Enteritidis, Heidelberg, and Hadar were attributed to poultry or eggs, whereas those due to serovars Typhimurium and Newport were associated with a wide variety of foods (Table 2).[9] Rare reports of *Salmonella* isolates in animals or animal feed does not preclude them from causing human outbreaks.

Table 2: List of top-ranked human *Salmonella* isolates (GERMS-SA, 2003–2015) and commonly reported animal, animal feed and environmental *Salmonella* isolates [7] in South Africa (2012–2014), compared with commonly-reported isolates from non-human sources in the USA, prior to the implementation of AGOA in 2016

Human isolates, RSA†	Animal isolates, RSA‡	Animal isolates, USA*
Typhimurium	√	√
Enteritidis	√	√
Isangi		
Infantis	√	√
Dublin		√
Heidelberg		√
Virchow		
Newport		√
Muenchen	√	√
Hadar	√	√
Anatum	√	√
Bovismorbificans		
Schwarzengrund	√	√
Arizonae		
Diarizonae		
Saintpaul		
Stanleyville		√
Irumu		
Braenderup		√
Agona		√

†Republic of South Africa

‡More than 180 isolates reported in the three-year period. Other isolates listed in column one above may be rarely reported.

*Data exclude outbreaks attributed to sources other than animals, such as leafy vegetables and fruit.

International food trade has made the monitoring of human, food and animal isolates of *Salmonella* mandatory so as to prevent international outbreaks. Complete serotyping is thus recommended on all *Salmonella* isolates due to a close association between the human and animal populations, in association with antimicrobial resistance monitoring.[7] The fact that known human pathogens may not occur commonly in animals, animal feeds and the agricultural environment suggests that more cohesive studies between human and veterinary surveillance programmes are mandatory.

The burden of foodborne disease will not decline without appropriate interventions regardless of whether foodborne disease is acquired from locally-acquired pathogens or from imported foodstuffs. Therefore, data concerning the prevalence of pathogens is critical to design appropriate interventions including good surveillance systems that link human, environmental and animal data.

Acknowledgements

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PLASMODIUM FALCIPARUM INFECTION OF LABORATORY STRAINS OF THE FOUR MAJOR AFRICAN MALARIA VECTOR SPECIES

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Introduction

The malaria parasite life cycle in the adult female *Anopheles* mosquito has received renewed attention in recent years with the development of transmission-blocking strategies.[1] These strategies can be targeted at any stage of the parasite's transmission cycle. The parasite life-stages in the vector, in particular ookinete formation in the midgut, with subsequent oocyst formation on the midgut wall, the release of sporozoites and their migration to the salivary glands of the mosquito, are important measurable variables in transmission blocking strategies. In order to evaluate these strategies, laboratory reared mosquitoes are artificially infected with the human malaria parasite, *Plasmodium falciparum*, by feeding them on infected blood using artificial membrane feeders.[2-4] One strategy is to add transmission-blocking-compounds (TBC) to the infected blood (asexual, stage IV of the parasite life cycle) prior to mosquito feeding and this is done through the standard membrane feeding assay (SMFA).[2] The TBCs may also be added to the gametocyte stages during culturing (Stages I-III), or by adding the compound to sugar water provided to the vector before, during and after infection feeding. In order to evaluate transmission blocking compounds, "Transmission Reducing Activity" (TRA) is measured. TRA is calculated by comparing the oocyst prevalence and oocyst numbers in the mosquito midgut (intensity) fed on infected blood with or without the TBC.[2, 5]

The Wits Research Institute for Malaria (WRIM) established a host-parasite infection facility in December 2014. Techniques to infect anopheline mosquitoes with *P. falciparum* have been optimized via successive infections of laboratory strains representing the four major African malaria vector species *Anopheles funestus*, *An. gambiae*, *An. coluzzii* and *An. arabiensis*. This study reports on the infection rates achieved in these four species. Furthermore, to test the hypothesis that adding a "compound" to mosquito feeding under laboratory conditions will result in a difference in oocyst intensity and/or prevalence[6], results following feeding of two of these species with the gentamicin and para-amino-benzoic acid (PABA) are given.

Materials and Methods

Parasite strain

A Green Fluorescent Protein (GFP-luc, NF54) *P. falciparum* strain was obtained from Dr. Mark Kennedy (Penn State University). This strain produces green fluorescent parasites through all stages of the life cycle.[7]

Plasmodium falciparum gametocyte culture

The NF54 *P. falciparum* strain was genetically modified[6] to create a NF54 GFP-Luc line that produces green fluorescent protein throughout the parasite lifecycle. This line was kindly donated by Dr. Mark Kennedy (Penn State University). Parasites were maintained *in vitro* at 37ºC in cell culture medium (Roswell Park Memorial Institute medium - RPMI-1640, Gibco®),

supplemented with 0.5% Albumax and 50mg/L hypoxanthine at ~6% haematocrit, and gametocytes were cultured according to standard protocols.[7] Briefly, on day 0 a gametocyte culture was seeded with 0.5-1% asexual parasitaemia and on day 3 the haematocrit was decreased to ~3%. Cultures were maintained for 16 days to ensure the development of stage V male and female gametocytes. On the day of infection the parasites were diluted with fresh red blood cells and serum to a final haematocrit of ~50% and stage V gametocytaemia of 0.6-1%. Care was taken to keep the parasites at 37ºC throughout the handling and feeding stages to prevent premature exflaggelation of male gametocytes. To estimate the viability of the parasites before feeding, an aliquot of the culture was incubated at room temperature for 10 min and visualised under light microscopy, which showed 0-2 exflagellation events per field.

Anopheles Mosquito preparations

Mosquitoes used in the optimization and other experiments were reared under standard insectary conditions (25-27°C, 80% relative humidity) and consisted of the following strains: *An. arabiensis* (KWAG; originating from northern KwaZulu-Natal, South Africa), *An. coluzzii* (G3 from the Gambia), *An. gambiae s.s.* (IANP20 from Nigeria) and *An. funestus* (FUMOZ from southern Mozambique).

Optimization experiments

In order to achieve infections with *P. falciparum* in all species/strains, a series of experiments of one replicate each per strain was conducted to optimize feeding success by varying the starvation periods prior to feeding. All strains were maintained on a 0.05% para-aminobenzoic acid (PABA) solution after emergence until dissection. The *An. arabiensis* sample was starved for 24 hours before feeding followed by a 72 hours starvation period after feeding. These mosquitoes were not provided with sucrose until 72 hours after feeding.[4] This increased feeding success and allowed sufficient time for all unfed females to die, limiting the amount of handling of the fed mosquitoes. The *An. funestus* sample was starved 72 hours before feeding, while the *An. gambiae* (IANP20) and *An. coluzzii* (G3) samples were starved 6 hours before feeding. Unfed females from all four strains were removed with an aspirator after feeding.

Infections of the *Anopheles* species/strains were conducted using the NF54 GFP-luc *P. falciparum* strain under laboratory conditions. Following the optimized starvation periods for each colony, samples of two to five day old adult females of each *Anopheles* strain (except for *An. funestus*, where eight to ten day old females were used because this age cohort tends to feed more prolifically), were offered *Plasmodium* gametocyte infected blood via a Hematek membrane feeder. The prevalence of oocysts (mean percentage of mosquitoes dissected presenting with oocysts) and the mean number of oocysts per specimen (intensity) were recorded for each experiment.

Effect of the antibiotic compound gentamicin on oocyst prevalence in An. arabiensis and An. coluzzii

To determine the effect of the antibiotic compound gentamicin compared with para-aminobenzoic acid (PABA) on oocyst prevalence and intensity, two to five day old, adult female mosquitoes from the KWAG and G3 strains were provided with a 10% sucrose solution containing either 0.05% gentamicin sulfate (40mg/ml) or 0.05% PABA (control) after emergence and after infection. Four biological repeats were conducted using *An. coluzzii* and three repeats using *An. arabiensis.* Starvation procedures were based on optimized minimum mortality before feeding, as described above. Infected bloodmeals were offered via glass feeders with hog membranes[4,6] at 37ºC for 30 minutes.

Dissection of mosquito midguts was performed on days 9 -10 after the infected blood meal to determine oocyst prevalence and intensity by means of fluorescent microscopy. Salivary glands were dissected 14 days post infection.[8] The salivary glands of each individual specimen were stained with Giemsa and viewed under phase contrast to determine sporozoite presence. Oocyst prevalence and oocyst intensity between gentamicin and PABA treatments were analyzed by means of unpaired non-parametric t-tests using Graphpad Prism (Version 7). Species differences with regards to oocyst counts were also compared for the two treatments.

Results & Discussion

Optimization experiments

Results from five baseline *P. falciparum* infection optimization experiments for each of *An. gambiae, An. arabiensis, An. coluzzii* and *An. funestus* showed that the prevalence of oocysts (mean proportion, expressed as a percentage, of mosquitoes presenting with oocysts) and the mean number of oocysts per specimen (intensity)[8] generally increased as the experiments were optimized over time. The feeding rate (proportion of mosquitoes that fed on *Plasmodium* infected blood) for *An. coluzzii* and *An. arabiensis* also increased over successive experiments (these trends were not recorded for *An. funestus* while the *An. gambiae* results were recorded only once because they were similar to the other two members of the *An. gambiae* complex – *An. arabiensis* and *An. coluzzii*). During the optimization experiments, the highest number of oocysts per specimen was found in *An. funestus*, while a steady increase in the number of oocysts per specimen was observed in *An. arabiensis* (KWAG).

Effect of the antibiotic compound gentamicin on oocyst prevalence in An. arabiensis and An. coluzzii

Following dissection of all test mosquitoes (treatment and control) and viewing under a fluorescent microscope, oocysts, if present, could be observed in the midguts of both *An. arabiensis* and *An. coluzzii* (Figure 1A), and sporozoites (if present) could be observed in the salivary glands (Figure 1B). Giemsa stained smears of the salivary gland content were used to confirm the presence of sporozoites (Figure 2). There was no statistically significant difference in oocyst prevalence between the PABA control (n=83) and the gentamicin treatment samples (n=82) in *An. coluzzii* ($p = 0.93$) and *An. arabiensis* (PABA: n=76; gentamicin: n=50; $p=0.17$).

Oocyst intensity (number of oocysts per midgut) was significantly higher in the gentamicin treated samples in *An. coluzzii* (n=82) and *An. arabiensis* (n=86) compared to their respective PABA controls (*An. coluzzii*, n=83; $p=0.01$; *An. arabiensis*, n=136, $p=0.02$) (Figure 3). However, the variance (F-test for variance) between the repeats in *An. arabiensis* differed significantly ($p<0.01$).

Non-parametric t-test comparisons of oocyst prevalence between species showed no significant differences between the PABA control cohorts ($p=0.2$) or between the gentamicin treatments ($p=0.7$). The same was true when oocyst intensities were compared between species (PABA: $p=0.64$ and gentamicin: $p=0.47$). These analyses indicate that either species can be used for infection purposes and that both species should respond similarly to treatments.

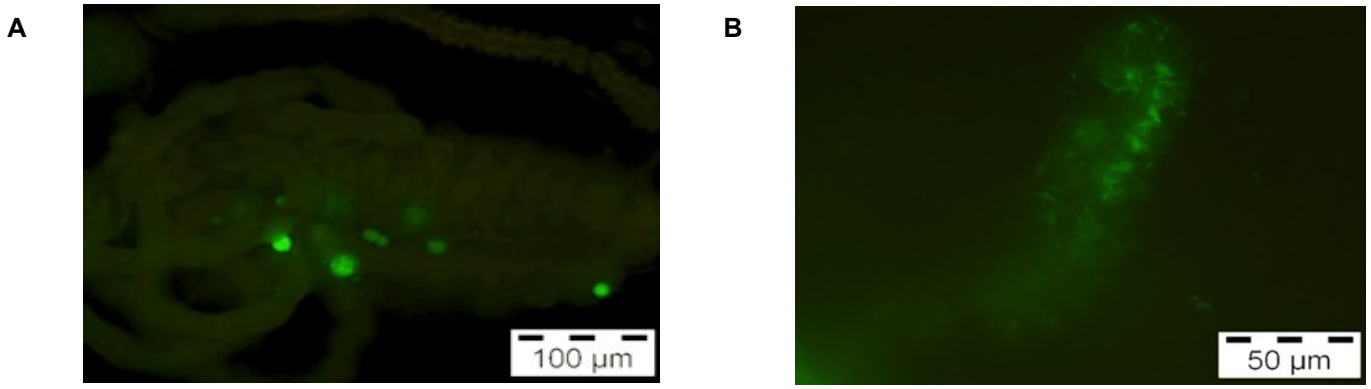

Figure 1: (A) Midgut of *Plasmodium falciparum* infected *Anopheles arabiensis* (KWAG) showing green fluorescent oocysts and (B) sporozoites in the salivary glands.

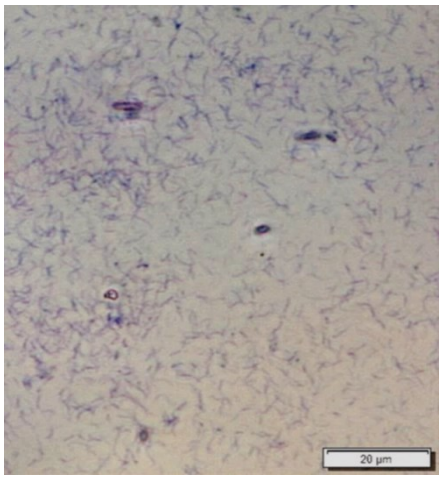

Figure 2: Giemsa stained *Plasmodium falciparum* sporozoites obtained from *Anopheles coluzzii* salivary glands (scale bar = 20um).

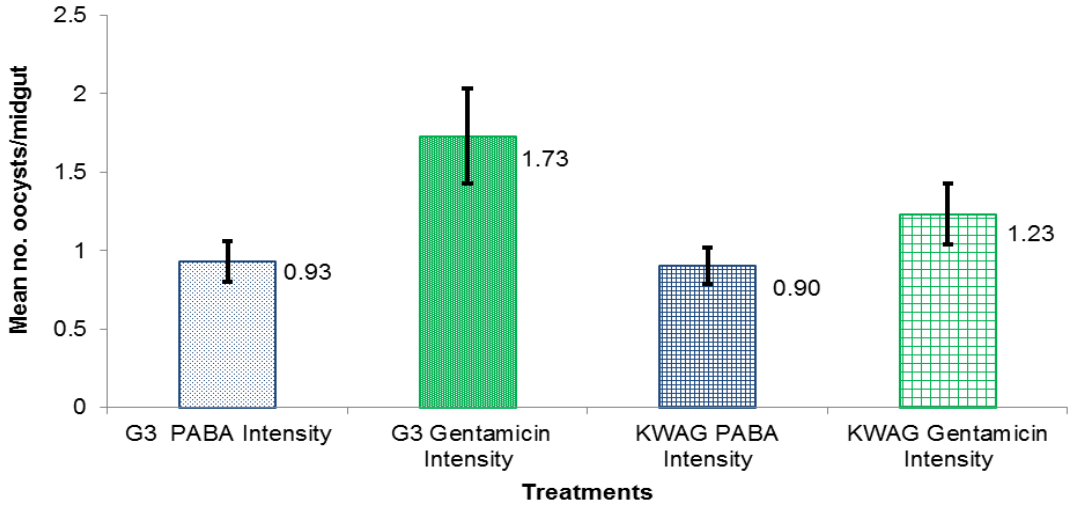

Figure 3: Oocyst intensities of *Plasmodium falciparum* infected samples of *Anopheles coluzzii* (G3) and *An. arabiensis* (KWAG) by gentamicin treatment and corresponding para-aminobenzoic acid (PABA) control.

Conclusion

All four major African malaria vector species were successfully infected with *P. falciparum* gametocyte cultures using artificial membrane feeders. *Anopheles funestus* presented with the highest number of oocysts of the four species. Gentamicin treatment increased oocyst intensity in both *An. coluzzii* and *An. arabiensis* and the positive sporozoite results confirmed that infection in these vectors can be achieved under laboratory conditions. This is the first time that *An. funestus* and *An. arabiensis* have been successfully infected in the laboratory with *P. falciparum.* The oocyst data confirmed that all four *Anopheles* species tested can be used in parasite infection studies.

The WRIM host-parasite infection facility is expected to enable the future testing of various novel anti-malarial drug compounds including transmission blocking vaccines using all four major African malaria vector species, thus providing an important operational research platform to support the control of malaria.

Acknowledgements

Staff of the Vector Control Reference Laboratory, NICD, are thanked for their assistance with the maintenance of mosquito colonies and preparing the mosquitoes for infection. This project is supported by the Strategic Health Innovation Partnerships (SHIP) Unit of the South African Medical Research Council with funds received from the South African Department of Science and Technology.

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TRANSMISSION ELECTRON MICROSCOPY AND THE PHENOTYPIC CHARACTERISATION OF PROKARYOTIC PATHOGENS: VISUALISATION OF BACTERIAL CAPSULES

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Introduction

The quality and interpretation of communicable disease surveillance data is underpinned by the diagnostic accuracy and depth of understanding of the pathogens involved. The Centre for Respiratory Diseases and Meningitis (CRDM) of the National Institute for Communicable Diseases (NICD) is therefore committed to the phenotypic and genotypic characterisation of respiratory/meningeal organisms that cause invasive disease.[1] Classically, pathogen invasive potential, as a manifestation of virulence through immune evasion, has been linked to the presence of a polysaccharide capsule[2-5], although, more recently, virulence through immune evasion has also been shown to be mediated by non-capsular antigens[6] conferring invasive potential to non-encapsulated isolates.[7-11]

The bacterial capsule is routinely used for typing of bacterial respiratory pathogens for diagnostic and surveillance purposes[2,12] and, as capsular polysaccharides elicit antigenic responses in the human host that confer type-specific immunity, they have been used successfully as vaccine targets and epidemiological markers.[6,12] The bacterial capsule lies external to the cell wall and is visible at the electron microscope level as a delicate network of fine, anastomosing polysaccharide fibrils.[13,14] Environmental factors that influence the production of bacterial extracellular polysaccharides include incubation temperature, composition and phase of the culture medium, position of cells within a colony, environmental oxygen concentration, growth phase of the organism and cell age.[13-19] Variation in capsule production is caused by point mutations in capsular genes, capsular gene inactivation due to insertion/deletion, changes in capsular gene expression involving phase variable genes, or loss of capsular genes.[2,7,8,20-22] In order to adequately characterise invasive non-typeable bacterial isolates, it is necessary to define the phenotypic variables and genotypic status of cells grown under defined culture conditions.

It is difficult to visualise extracellular capsular material using transmission electron microscopy (TEM), given the delicate composition of highly hydrated polymers of monosaccharides or heteropolysaccharides.[4,13,23] Electron microscopists have addressed this challenge by using a variety of fixation/immuno-electron microscopy protocols.[6,8,13,24-26] However, the majority of TEM studies have involved the use of cationic dyes in conjunction with fixatives in order to simultaneously stain and stabilise the anionic capsular material. With an additional amine or diamine, the most frequently used dyes have been ruthenium red and alcian blue, with or without *en bloc* staining with tannic acid, uranyl acetate or lanthanum nitrate.[13,15,27-29] As some of the variation in capsular staining success for TEM is attributable to differences in cell wall composition between Gram-positive and Gram-negative bacteria[30], TEM of Gram-negative bacteria tends to involve the use of alcian blue[13,28,31] with different types of alcian blue providing different staining intensities.[32] In terms of interpretation of bacterial capsule staining, it is important to note that alcian blue staining is frequently non-specific[13] and that the fragile nature of the capsule can result in the apparent absence of capsular material in up to 70% of encapsulated cells, after TEM processing with any cationic dye.[31]

A variety of different preparative protocols (both published and modified) were assessed by the Electron Microscope Laboratory of the NICD, for staining of capsules of Gram-positive and Gram-negative bacteria, with the ultimate aim of describing the ultrastructure of selected non-typeable bacterial isolates identified during national laboratory-based surveillance for invasive bacterial respiratory pathogens.

Methods

American Type Culture Collection (ATCC) isolates of *Bordetella pertussis, Haemophilus influenzae, Neisseria lactamica* (non-encapsulated Gram-negative control), *N. meningitidis* and *Streptococcus pneumoniae* (R6 as a non-encapsulated Gram-positive control) were used to develop and compare various protocols. The two protocols that produced the most consistent and comparable capsular imaging are presented in Figure 1, with the cationic dyes of choice being ruthenium red (Sigma-Aldrich CAS# 11103-72-3) and the pyridine variant of alcian blue (Sigma-Aldrich CAS # 123439-83-8), together with the diamine L-lysine acetate. Discs from 'young' colonies containing exponentially growing organisms were punched out of agar plates and the colonies processed *in situ* until graded ethanol dehydration at which point they were scraped off and pelleted by gentle centrifugation (4^0C, 300 x g, for 30 minutes). It proved necessary to osmotically adjust the 0.1M sodium cacodylate buffer, which was accomplished by the addition of 0.09M sucrose, 0.01M $CaCl_2.H_2O$ and 0.01M $MgCl_2.6H_2O$, as described by Hammerschmidt *et al.*[15]

Results and Discussion

Comparison of isolates fixed with either ruthenium red or alcian blue-pyridine variant (Figures 2a-h & 3a-f) indicated that ruthenium red plus diamine staining was adequate for visualizing Gram-positive cells and many Gram-negative cells, whilst some of the Gram-negative cells required dual processing with both selected dyes (Figure 3g,h), as alcian blue stains homopolymeric, non-sialylated capsular material - which does not stain with ruthenium red. However, alcian blue also stained all glycoproteins indiscriminately (Fig. 4a), and the scraping and subsequent pelleting of fixed cells with surface films from the agar plates produced some anomalous images in terms of homogeneity in capsule visualisation within a single sample (Figures 4b,c). If obtainable, the crisper outlines of ruthenium-red stained capsular material are preferable to the somewhat amorphous outkines of alcian blue, for relative capsule comparison between isolates.

Studies involving selected organisms and protocols have been successfully performed using numerous surveillance isolates, with excellent concordance between the phenotypic and genotypic characteristics of the *N. meningitidis* isolates and most of the *S. pneumoniae* isolates examined thus far (Figures 4d,e, f-h & 5a). Discordant observations may be due to the age and culturing history of the isolate and/or the fact that whole genome sequencing studies were completed months before the isolate was re-cultured for electron microscopy processing.

Several cultures of invasive non-typeable *S. pneumoniae* isolates were found to have definite fringes of exopolysaccharides suggestive of capsule (Figure 5b). These were all in conjunction with a bacteriophage infection (Figure 5b-g). The exciting research avenues down which this observation could lead are beginning to be explored, and although the literature on pneumophages is extensive, capsule presence has not been documented previously – possibly because of the current global reliance on molecular characterisation alone. It is therefore hoped that the multi-disciplinary approach of the NICD to the study of these pathogens will provide important information in this regard.

Acknowledgements

Professor Anne von Gottberg is thanked for her support. This work was funded by the National Institute for Communicable Diseases.

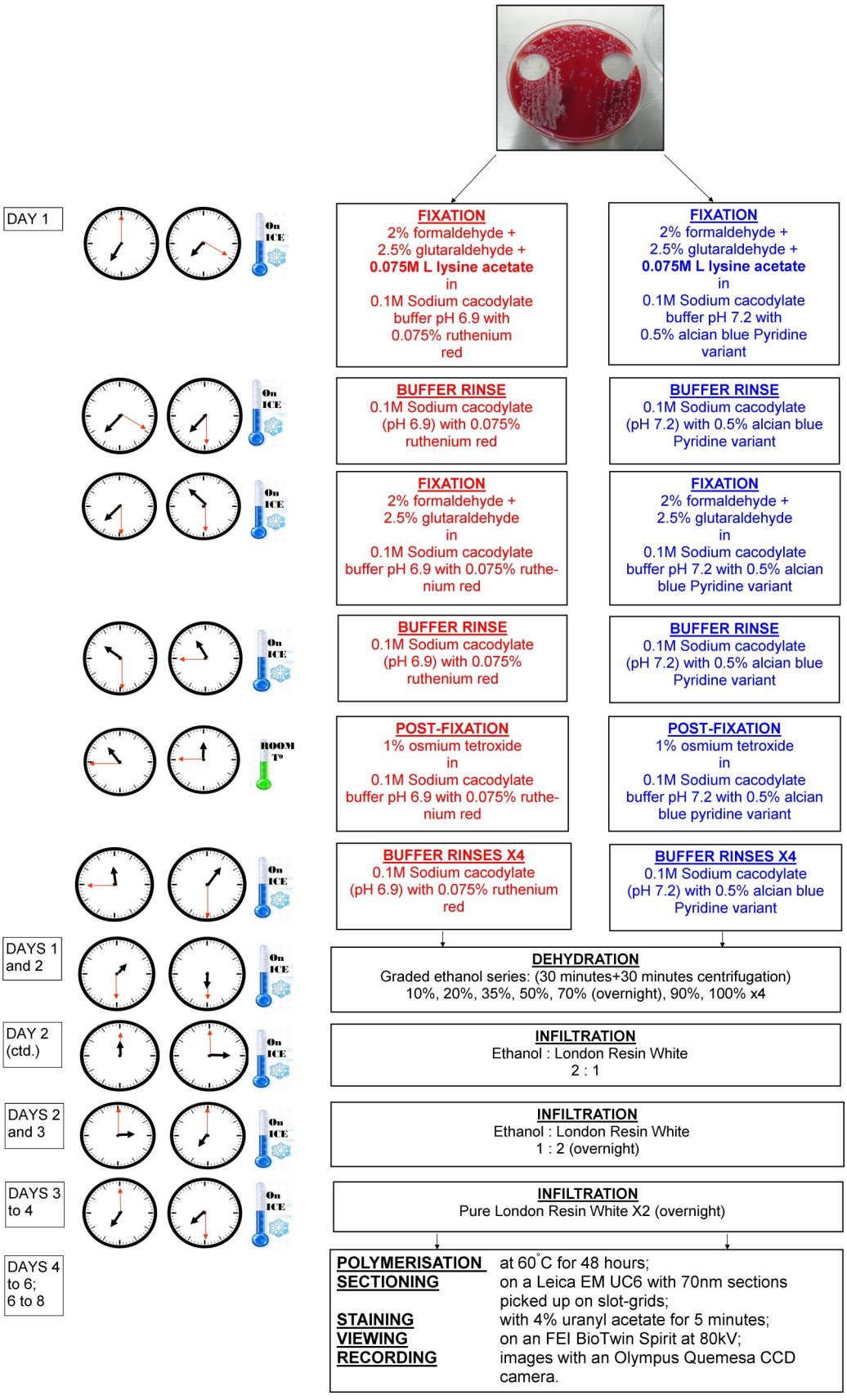

Figure 1: Schematic of the Transmission Electron Microscopy protocols developed for the visualisation of bacterial capsules.

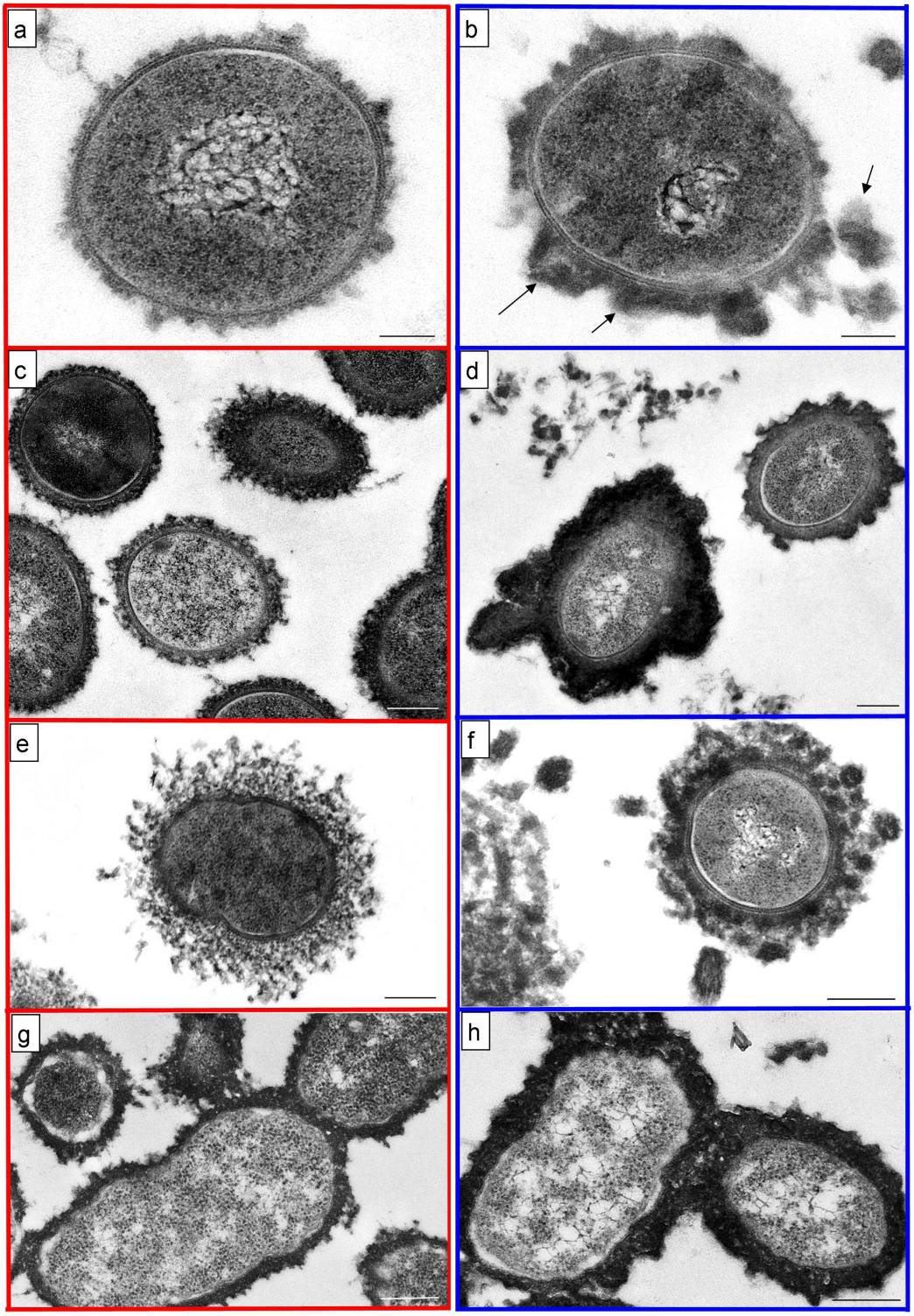

Figure 2: American Type Culture Collection isolates processed with L-lysine acetate and either ruthenium red (a,c,e,g) or the pyridine variant of alcian blue (b,d,f,h), illustrating exo-polysaccharide staining in both Gram-positive cells (a-f) and in Gram-negative cells (g,h): (a,b) *Streptococcus pneumoniae* R6 (laboratory-constructed, non-encapsulated strain) showing more non-specific staining with alcian blue (arrows) than with ruthenium red; (c,d) *S. pneumoniae* serotype 1, with a capsular crystalline filigree (c), and a densely staining pleomorphic capsular zone (d); (e,f) *S. pneumoniae* serotype 19F with a delicately stained capsule (ruthenium red) and a globular, dense staining of exo-polysaccharides (alcian blue); (g,h) *Haemophilus influenzae* type b in which either staining method works well, with a thicker capsular zone apparent with alcian blue staining. Scale bars: a, b = 0.1 µm; c - h = 0.25 µm.

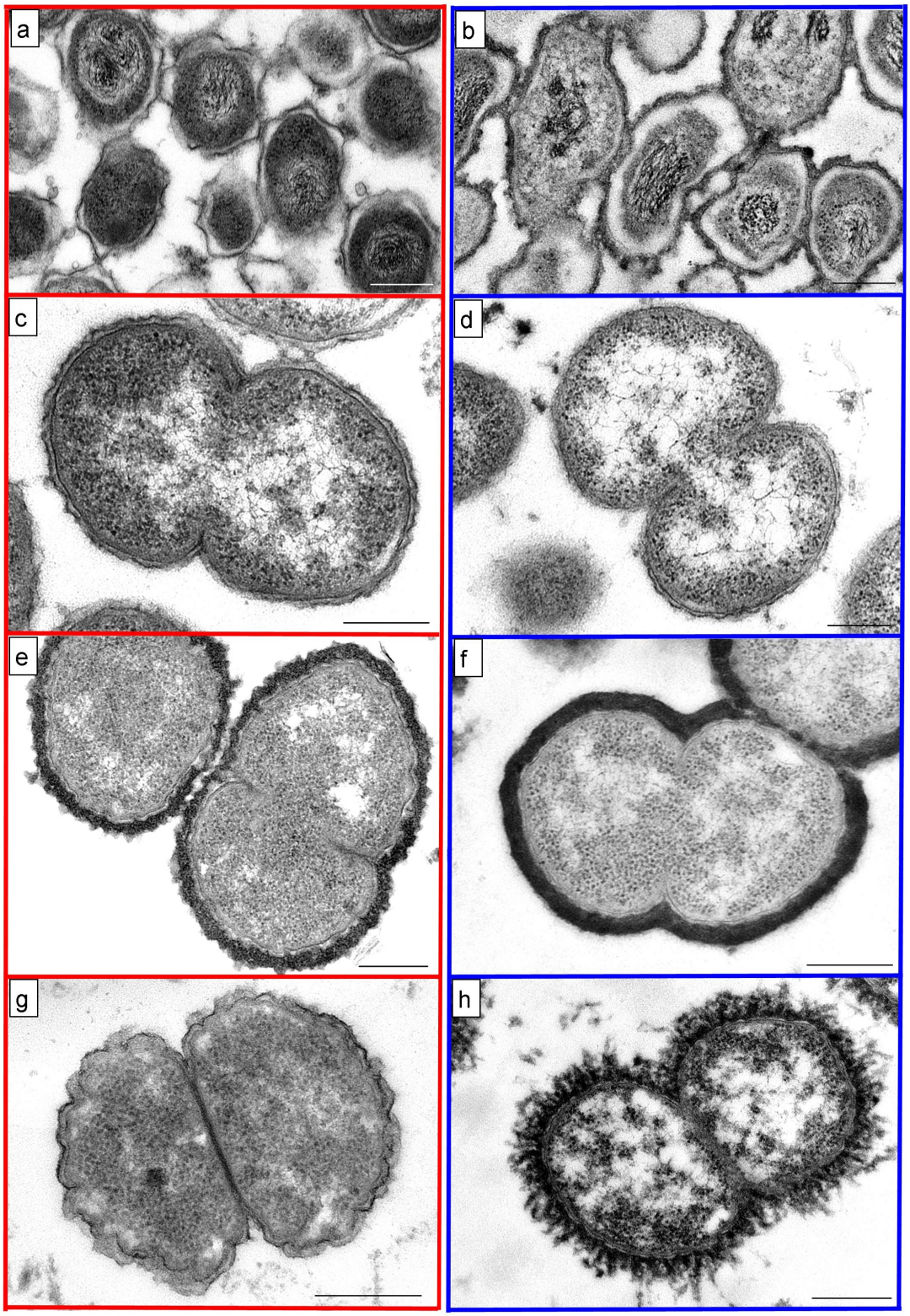

Figure 3: Gram-negative cells (ATCC) processed with L-lysine acetate and stained with either ruthenium red (a,c,e,g) or the pyridine variant of alcian blue (b,d,f,h): (a,b) *Bordetella pertussis* in which there is little evidence of capsule when stained with ruthenium red, but a distinct glycocalyx with the alcian blue staining; (c,d) *Neisseria lactamica* clearly lacks any capsular staining with either dye, corroborating genetic data that it is acapsulate; (e,f) *N. meningitidis* serogroup W in which capsule is evident after either staining protocol, but differing in texture – the ruthenium red is hard and crystalline, the alcian blue staining is softer and less structured; (g,h) *N. meningitidis* serogroup A in which no capsule is evident after ruthenium red staining, but obvious with alcian blue staining. All scale bars = 0.25µm.

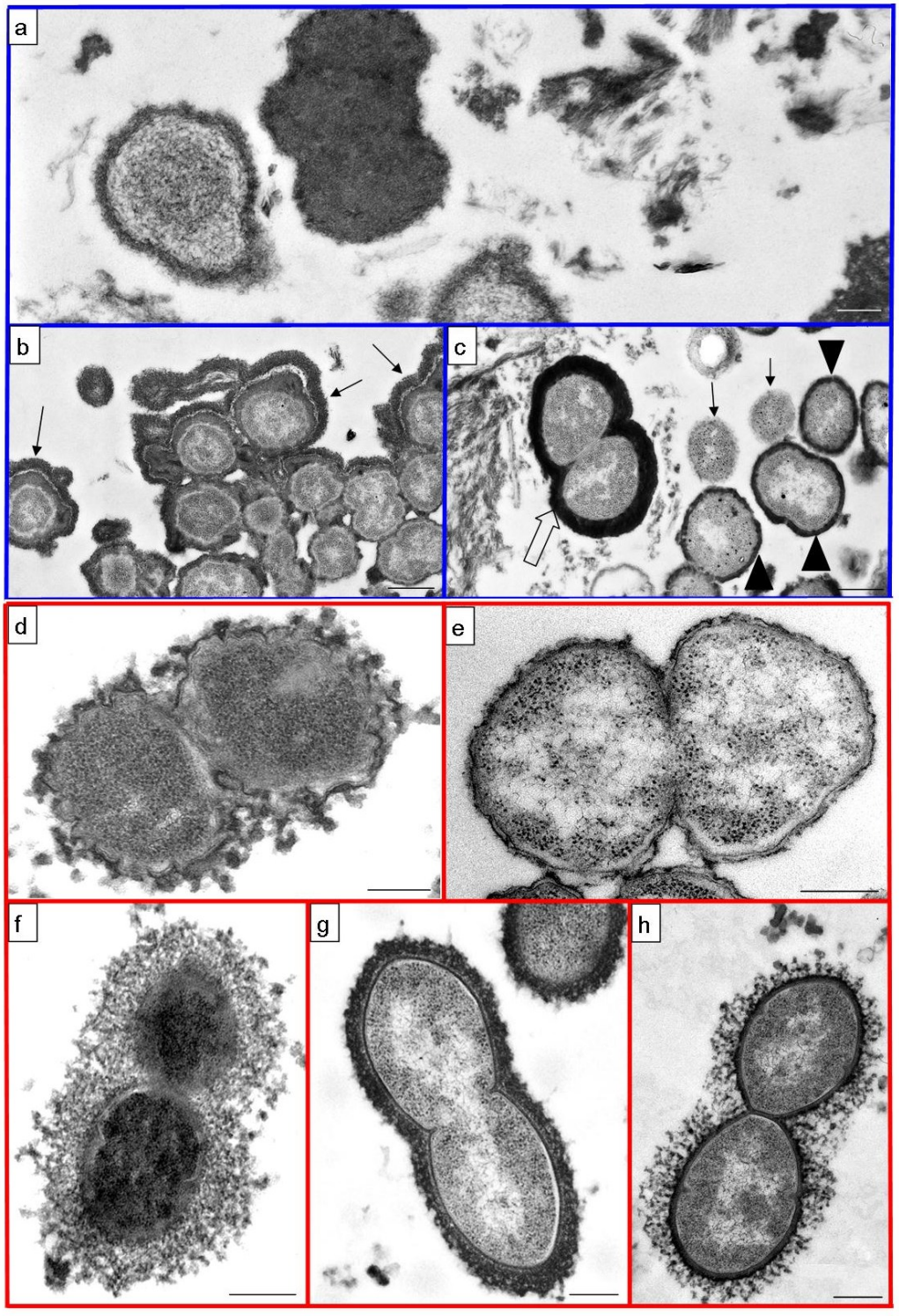

Figure 4: Alcian blue pyridine variant staining variation (a-c), and application of ruthenium red staining to genotypically characterised CRDM surveillance isolates (d-h): (a) Non-specific staining of glycopolysaccharides and mucopolysaccharides with Alcian blue is evident in this section through the culture plate agar; (b) positional variation in staining of *Neisseria meningitidis* serogroup W cells, with peripheral cells (arrows) having extensive exopolysaccharide layers, but cells towards the centre of the colony showing less of this non-specific staining; (c) *N. meningitidis* serogroup W cells demonstrating the entire range of capsular staining from nothing (arrows) to discrete (arrowheads) to enormous (open arrow); (d) encapsulated surveillance isolate of *N. meningitidis* serogroup E; (e) *cnl* (capsule null locus) *N. meningitidis* surveillance isolate; (f) *Streptococcus pneumoniae* serotype 19F; (g) *S. pneumoniae* serotype 1; (h) *S. pneumoniae* serotype 8. Scale bars: a, d, e f, g, h = 0.25µm; b, c, = 0.5 µm.

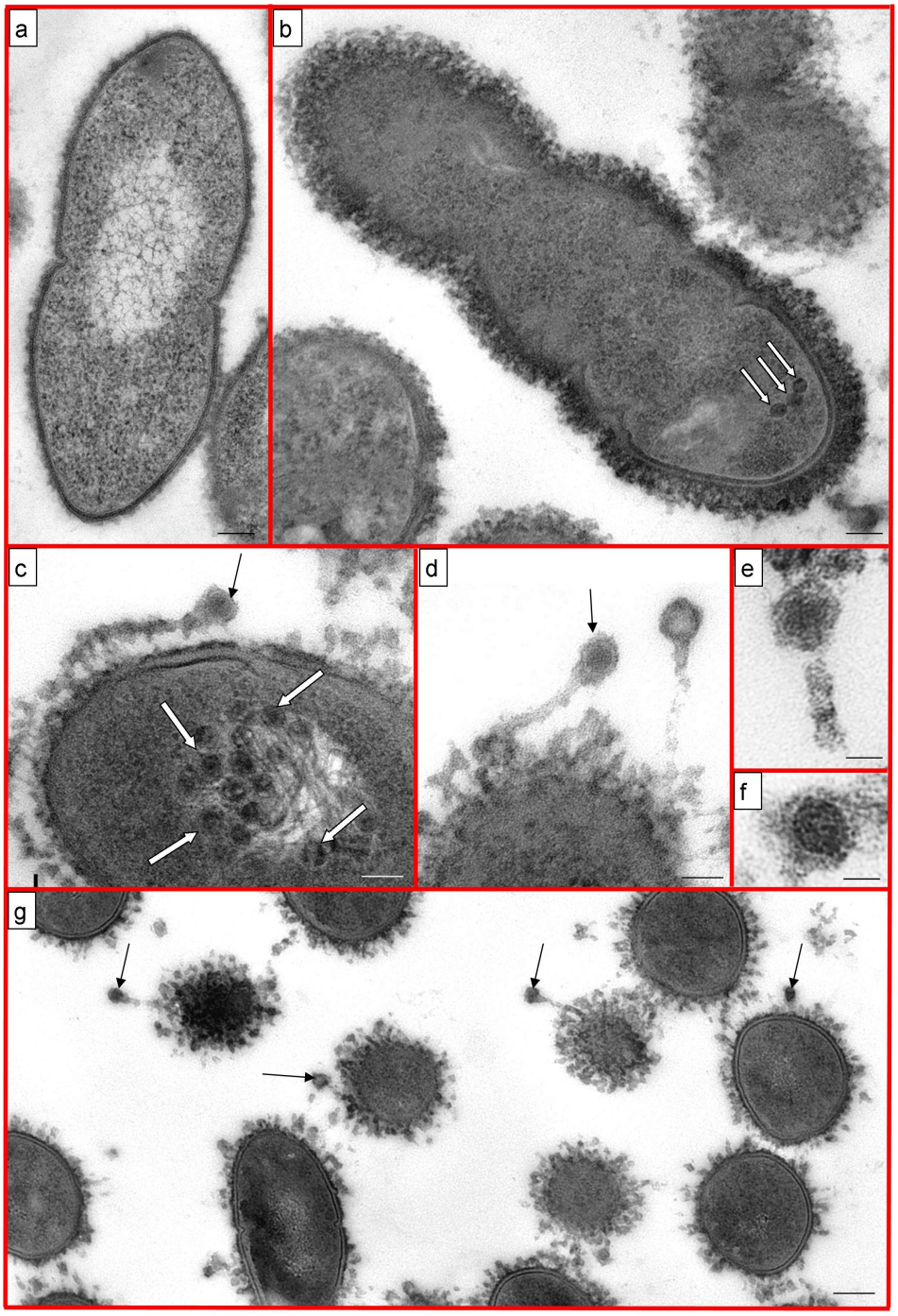

Figure 5: Ultrastructural characterisation of *Streptococcus pneumoniae* surveillance isolates, using ruthenium red plus L-lysine acetate protocol: (a) non-encapsulated cell confirms molecular characterisation of this isolate; (b) whole genome sequencing indicated that this isolate lacked a capsule – although TEM revealed the presence of a dense, outer layer of polysaccharides. Note the bacteriophage profiles (arrows) within the cytoplasm; (c) genetically characterised isolate that should lack capsule, but which has an outer fringe of stained material as well as an active bacteriophage infection – viral nucleocapsids (white arrows) can be seen tangled within the bacterial nucleoid and a bacteriophage (black arrow) can be seen associated with the extracellular layer; (d) two bacteriophages, one with an apparently empty nucleocapsid (arrow) penetrating the outer stained layer of a cell from the same culture as in (c); (d) Siphoviridae-like pneumophage from another visually-encapsulated isolate; (e) empty nucleocapsid illustrating the capsomers (scalloped outer edging); (f) low power view of a genetically non-encapsulated isolate, with visible capsular-like material around the cells which are infected by bacteriophage (arrows).Scale bars: a,b = 125nm; c,d = 40nm; e,f = 20nm; g = 100nm.

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Table 1: Provisional number of laboratory confirmed cases of diseases under surveillance reported to the NICD - South Africa, corresponding periods 1 January - 31 March 2015/2016*

Disease/Organism	1 Jan to 31 Mar, year	EC	FS	GA	KZ	LP	MP	NC	NW	WC	South Africa
Anthrax	2015	0	0	0	0	0	0	0	0	0	0
	2016	0	0	0	0	0	0	0	0	0	0
Botulism	2015	0	0	1	0	0	0	0	0	0	1
	2016	0	0	0	0	0	0	0	0	0	0
Cryptococcus spp.	2015	182	60	294	389	45	80	11	91	195	1347
	2016	194	51	405	321	93	113	9	93	86	1365
Haemophilus influenzae, invasive disease, all serotypes	2015	3	4	14	12	0	4	0	0	33	70
	2016	7	3	17	3	1	2	2	2	13	50
Haemophilus influenzae, invasive disease, < 5 years											
Serotype b	2015	0	1	1	0	0	0	0	0	2	4
	2016	1	1	2	0	1	0	0	1	0	6
Serotypes a,c,d,e,f	2015	0	1	0	2	0	0	0	0	1	4
	2016	0	1	2	0	0	0	0	0	0	3
Non-typeable (unencapsulated)	2015	0	0	2	1	0	0	0	0	3	6
	2016	0	0	3	0	0	0	0	0	0	3
No isolate available for serotyping	2015	1	1	2	4	0	1	0	0	2	11
	2016	1	1	3	1	0	0	0	0	1	7
Measles	2015	0	0	0	0	0	0	3	1	1	5
	2016	0	0	0	2	0	1	0	1	0	4
Neisseria meningitidis, invasive disease	2015	7	2	2	4	0	0	0	1	6	22
	2016	2	0	5	3	0	0	0	1	7	18
Novel Influenza A virus infections	2015	0	0	0	0	0	0	0	0	0	0
	2016	0	0	0	0	0	0	0	0	0	0
Plague	2015	0	0	0	0	0	0	0	0	0	0
	2016	0	0	0	0	0	0	0	0	0	0
Rabies	2015	0	0	0	1	1	0	0	0	0	2
	2016	0	0	0	1	0	0	0	0	0	1
Salmonella typhi	2015	1	0	7	5	0	2	0	0	4	19
	2016	0	0	21	5	4	1	0	1	17	49
Streptococcus pneumoniae, invasive disease, all ages	2015	29	32	148	82	12	17	5	17	143	485
	2016	39	34	137	50	17	11	7	13	118	426
Streptococcus pneumoniae, invasive disease, < 5 years	2015	5	3	21	17	1	3	1	4	18	73
	2016	4	3	34	5	5	0	1	1	20	73
Vibrio cholerae O1	2015	0	0	0	0	0	0	0	0	0	0
	2016	0	0	0	0	0	0	0	0	0	0
Viral Haemorrhagic Fever (VHF)											
Crimean Congo Haemorrhagic Fever (CCHF)	2015	0	0	0	0	0	0	0	0	0	0
	2016	0	0	0	0	0	0	0	0	0	0
Other VHF (not CCHF)	2015	0	0	0	0	0	0	0	0	0	0
	2016	0	0	0	0	0	0	0	0	0	0

Footnotes

*Numbers are for cases of all ages unless otherwise specified. Data presented are provisional cases reported to date and are updated from figures reported in previous bulletins.

Provinces of South Africa: EC – Eastern Cape, FS – Free State, GA – Gauteng, KZ – KwaZulu-Natal, LP – Limpopo, MP – Mpumalanga, NC – Northern Cape, NW – North West, WC – Western Cape

0 = no cases reported

Table 2: Provisional laboratory indicators for NHLS and NICD, South Africa, corresponding periods 1 January - 31 March 2015/2016*

Programme and Indicator	1 Jan to 31 Mar, year	EC	FS	GA	KZ	LP	MP	NC	NW	WC	South Africa
Acute Flaccid Paralysis Surveillance											
Cases < 15 years of age from whom specimens received	2015	27	9	18	27	10	15	1	5	6	118
	2016	9	4	23	29	10	18	8	1	9	111

Footnotes

*Numbers are for all ages unless otherwise specified. Data presented are provisional numbers reported to date and are updated from figures reported in previous bulletins.

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Monitoring for the presence of polio in a country is based on AFP (acute flaccid paralysis) surveillance – the hallmark clinical expression of paralytic poliomyelitis. The clinical case definition of AFP is an acute onset of flaccid paralysis or paresis in any child under 15 years of age. AFP is a statutory notifiable disease and requires that 2 adequate stool specimens are taken as soon as possible, 24 to 48 hours apart, but within 14 days after onset of paralysis, for isolation and characterisation of polio virus. The differential diagnosis of AFP is wide, the most common cause of which is Guillain-Barre Syndrome. The incidence of AFP in a population has been studied in a number of developing countries and WHO have determined, as a result of these studies, that the criterion for adequate surveillance of AFP is 4 cases per 100 000 population of children less than 15 years of age (it was formerly 1 per 100,000 but this was thought to be inadequately sensitive).

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