BACTERIOLOGICAL TRACING OF*Haemophilus influenzae*IN SOME PUBLIC TOILET SEAT BOWLS OF PORT HARCOURT

ABSTRACT

The study aimed at tracing *Haemophilus influenzae* on toilet seat bowls of some public toilets inPort Harcourt. The toilets were codedDER, TYU, AFR for park, market and hospital toilets respectively,for the purpose of the study.Swabs were collected aseptically from the toilet seat bowls, with a moistened swab stick, and then introduced into a sterile prepared peptone water for viability of *Haemophilus influenzae*. Thereafter, the spread plate technique was adopted in which theswabbed samples were inoculated into Chocolate agar. Result showedthe mean load of *Haemophilus influenzae* on the toilet seat bowl. Toilet coded DER had a*Haemophilus influenzae* mean count of 5 x 10^2CFU/ml while codes TYU and AFR had a mean counts of 6 x 10^2 and 9 x 10^2 CFU/ml respectively. A percentage frequency occurrence of *Haemophilus influenzae* on the toilet bowl showed DER had 17% while codes TYU and AFR had 25 and 58% occurrence respectively. A total of 49 isolates of *Haemophilus influenzae*were identified macroscopically and biochemically.Thus, the presence of*Haemophilus influenzae*on surface of toilet seat bowl may have resulted from aerosols generated during flushing. Hence, influenza infection (respiratory tract infection) may affect users.Due to aerosilization after flushing feces, toilet seats should be closed before and after flushing at all times specifically, toilets of code AFR.

Keywords:*Haemophilusinfluenza,* Seat Bowls,Public Toilet, Port Harcourt

INTRODUCTION

Fecal defecation in toilets comes up with numerous challenges as microbes are expelled, specifically bacteria (Franks et al., 1998). According to Palmer (1981), the bacterium, *Haemophilus influenzae*is amongst many bacteria that areexpelled in human waste. The bacterium is a normal floral of the human body were they colonize the upper respiratory tract (nose and mouth) as commensals. *Haemophilus influenzae* are implicated in respiratory tract infection as it could be transmitted from person to person by inhalation of droplets (Johnston & Apicella, 2009). Thus, *Haemophilus influenzae* presence on toilet seat may spark up respiratory challenges considering the bacterium aerosolized property and the high magnitude of aerosols in the toilet(Johnston & Apicella, 2009). Abney (2021) stated that toilet is as a receptacle were humans urinate or defecate and thereby meet their sanitary needs.A toilet consists of the bowl,

seat cover, the tank, showere points etc. the toilet bowl has a lid cover which covers the bowl toilet when not in use, to prevent small items from falling in, or to reduce the spread of germs and odour when water splashes from the toilet on flushing.In most toilets the lid is absent or even when present is left open (Abney, 2021). Public toilets are now made available for public use. The public toilets areessential and made available to address the sanitation-related target of the Millennium Development Goals (Moreira et al., 2022).Public toilet may be located in the markets, schools, eateries, offices, factories, hospitals, factories etc. Lack of public toilets in a public vicinity calls for open defecation which is associated with offensive odour from feces and urine. Open defecation in-turn disperse fecal matter, degrading air quality for diseases emergence via inhalation (Moreira et al., 2022). Large concentrations of bacteria and virus are found in stool after defecation and in the process of flushing after use, these bacteria disperse onto external parts of the toilet, such as the seat bowl sides, bowl rim and bowl seat (Gardner, 2004). Bacteria such as *Escherichia coli*,*Haemophilus influenzae Salmonella*sp. and *Shigella*sp.of less than 10^6 coliform forming units are often dispersed and remain under conditions of desiccation on toilet seatsfor an optimum nine(9) days respectively for the stated bacteria (Saleem et al., 2019). According to Knowlton et al. (2018) there have been reports of outbreaks of diseases associated with flush toilets. Flush toilets have been implicated in the spread of diseases via aerosols generated. Outbreak such as Shigellosisamongst many others have been reported.Although measures adopted to reduce the incident of pathogen in toilets have been adopted. The presence of *Haemophilus influenzae* on toilet seat other than inside the toilet bowl may pose respiratory challenges and direct transmission into human body. Thus, the study aimed at tracing of *Haemophilus influenzae* on toilet seat bowls.

MATERIALS AND METHODS

Study Area

Three classes of public toilets (hospital, market and park toilets) were chosen for the study. The toilets were coded AFR, TYU and DER for hospital, market and park respectively, for the purpose of the study. The code AFR is unique, with users mainly patients and out-door patients while code TYU and DER users are largely for general use. All servicing public toilet points are located in Port Harcourt, Nigeria.

Collection of Swab from the Seats of Toilet Bowl

Swabs from the toilet seat bowl were collected using a swab stick in the mornings when the toilets have been sanitized for early users.The swabbed samples were collected aseptically, with a moistened swap stick, and then introduced into a sterile prepared peptone water in a tube for increased visibility of *Haemophilus influenzae*(Ramadi &Asgharian 2020). The swabbed samples after collection werethen taken to the Biology Laboratory of Ignatius Ajuru University of Education, Rumuolumeni for Microbiological analysis

Determination of*Haemophilusinfluenzae* Load

Enumeration of *Haemophilusinfluenzae* load on the toilet seat bowl involved employing the spread plate technique as adopted by Cheesebrough (2006). The technique involved the spread of an aliquot of 10^{-1} obtained from the dilution of the swabbed sampleintroduced in peptone water. The aliquote was inoculated on a freshly prepared chocolate media. This was then followed by spreading, with a glass spreader and incubation at 37^{o}C for 24 hours. Growth observed were identified and counted or enumerated as colony forming unit per ml.Chocolate media used was prepared by theaddition of five mill blood into a sterile hot prepared Nutrient agar,a general purpose agar for the growth of non-fastidious microorganism. Prior to the addition of the blood,

the Nutrient agar media was prepared by dissolving the required quantity into distilled water, autoclaved as instructed by manufacturer before use.

Biochemical Characterization of*Haemophilus influenzae*

In presumptive test to characterize *Haemophilus influenzae*, certain morphological features were considered, with respect to size, shape, colour, elevation and opacity of the isolate/colony on the media plate. In further test to confirm the identity of *Haemophilus influenzae*, Some key biochemical reactions were adopted such as the Methyl red test, Citrate test ,Voges-Proskauer etc.

Methyl Red/ Voges-Proskauer Test

In carrying out this test as adopted by Cheesebrough (2006), a loopful of the *Haemophilus influenzae* was inoculated into 10ml sterile MR/VP broth medium, prepared according to manufacturer's instructions. The tube was then incubated at 35 -37^{0}C for 48 hrs, after incubation, the broth culture (*Haemophilus influenzae)* was shared into two parts (5ml) each, one part represented the methyl red test while the other part represents the Voges Proskauer test. To the part with methyl red test, 5-6 drops of methyl red reagent was added and to the part with Voges Proskauer test, 0.6ml (6 drops) of 5% a – naphthol and 0.2ml (2 drops) of 40% KOH was reagent added. Development of bright red coloration is indicative of positive MR/VP test, thus confirms*Haemophilus influenzae*and a reverse indicative of a non-*Haemophilus influenzae*.

Citrate Test

The test was done to determine the ability of*Haemophilus influenzae* to utilize Sodium Citrate as its source of carbon and inorganic ammonium salt as its source of nitrogen accordingly as done

by Cheesebrough (2006). Simmon citrate agar was prepared in a capped tube and a sterile wire loop used to pick a loopful of the presumed *Haemophilus influenzae* and streaked on slant surface. The tube was then incubated at 37^0C for 24hrs. Change in colour from green to blue indicated a positive result for *Haemophilus influenzae* while no change in colour indicated a negative*Haemophilus influenzae* result (Cheesebrough, 2000).

Indole Test

The test was carried out to ascertain the ability of *Haemophilusinfluenzae*to breakdown the amino acid tryptophan in the medium into indole in the presence of the enzyme tryptophanase. *Haemophilus influenzae* isolates were inoculated into test tubes containing 10ml of sterile tryptone broth and the tube incubated for 24 hours at 37^oC. Thereafter, 0.5ml of Kovac's reagent was added to the media and shaked gently and examined for red colour in the surface layer which indicates a positive result. No colour change, indicates a negative result(Cheesebrough, 2000).

Catalase Test

In a follow up to this text, to determines if *Haemophilus influenzae*possess the enzyme catalase, this enzyme is able to catalyze the reaction. The test was done to determine the ability of *Staphylococcus aureus* to breakdown Hydrogen Peroxide into Oxygen and water. In achieving this, a visible effervescent denoted *Haemophilus influenzae* positive when a small inoculum was introduced into a 3% hydrogen Peroxide solution placed on a slide, while the absence of catalase is evident by a lack of or weak production of effervescence or non-*Haemophilus influenzae* (Cheesebrough, 2000).

Sugar Fermentation Test

This sugar test was done to evaluate the ability of the *Haemophilus influenzae* to utilize sugar(lactose and maltose) to produce acid and gas. Peptone broth (1%) incorporated with 1% sugar was used to constitute the sugar broth test. An indicator was added to the sugar medium with Durham tube added in the tube in an inverted position. After sterilization, a loopful of the *Haemophilus influenzae* was introduced into the test tubes and then incubated at 35 -37^{0}C for 24 - 48 hrs. Change in color from purple to yellow and gas production indicated a positive sugar fermentation test for *Haemophilus influenzae* while no change in color depicted no sugar utilization (Cheesebrough, 2000).

Motility Test

This test was used to determine the motility of the *Haemophilus influenzae*. Motility of an organism is determined by the presence of flagella (a locomotory organelle). Semi solid Nutrient agar was used for this test. The media was prepared and the isolate picked with a sterile straight wire into it by stabbing. Thereafter, the medium in the tube was incubated at 37^{0}C for 24 - 48 hrs. Growth in diffuse form from the line of stab into the medium indicated a positive result, whereas growth only along the line of stab indicated a negative result (Cheesebrough, 2000).

Morphological Characterization

Gram Staining

Gram staining reaction was done as carried out by Cheesebrough,(2000)to confirm that the isolates Gram status. A smear was made by placing a drop of distilled water on a clean grease-

free slide using sterilized wire loop, loopful of 18-24 hours old culture of bacteria was mixed with the drop of sterile distilled water on the slide and spread evenly. The smear was air-dried and fixed by passing the slide several times through a slow burning flame of the Bunsen burner. Crystal violet stain was applied on the smear and allowed to remain on the slide for 1 min. The crystal violet was rinsed off under slow running tap water. Lugol's iodine (a mordant) was used to cover the slide for 1min. Distilled water was again used to rinse off the iodine, alcohol was used for 30 second to decolorize the smear before rinsing with water. Smear was counterstained with safranin for 30 seconds and was then washed with water. Blot dried and examined under x100 objective lens.

RESULTS

Enumeration of*Haemophilus influenzae*Loadon Toilet Seat Bowl

Table 1, showed the mean load of *Haemophilus influenzae* on the toilet seat bowl. Toilet coded DER had a*Haemophilus influenzae* mean count of 5×10^2CFU/ml while points coded TYU and AFR had amean counts of 6×10^2 and 9×10^2 CFU/ml respectively.

Table 1; Mean Load of*Haemophilus influenzae*

Bacteria	**DER (CFU/ml)**	**TYU(CFU/ml)**	**AFR(CFU/ml)**	**T-test**
Haemophilus influenza	5×10^2	6×10^2	9×10^2	$P>0.05$

Keys;

(CFU/ml) = Coliform Forming Unit Per Mill, **DER**=Park,**TYU**=Market, **AFR**=Hospital

Percentage Frequency of Occurrence of *Haemophilus influenzae*

Table 2, showed the percentage frequency occurrence of *Haemophilus influenzae* on the toilet bowl. Sample point DER had 17 percentage occurrence while sample points TYU and AFR had 25 and 58 percentage occurrence respectively.

Table 2; Percentage Frequency of Occurrence of *Haemophilus influenzae*

Toilet Codes	Frequency of Occurrence (n)	% Frequency of Occurrence
DER	2	17
TYU	3	25
AFR	7	58

Keys; **DER**=Park,**TYU**=Market, **AFR**=Hospital

Macroscopic Feature of*Haemophilus influenzae*

Table 3, showed the macroscopic features of the *Haemophilus influenzae* colony on Chocolate agar media as seen on plates after inoculation and incubation. The colonies were noted cream white, with a very small sized colony. The colonies were also noted oval in shape, opaqued with low elevation.

Table 3; Macroscopic Feature of*Haemophilus influenzae*

Gram Reaction	Colour	Size	Shape	Opacity	Edge	Elevation
	White	Small	Oval	Opaque	Curved	Low

Biochemical Feature of*Haemophilus influenzae*

Table 4, showed the biochemical characterization of *Haemophilus influenzae* as expressed. *Haemophilus influenzae* utilized lactose, glucose, and also indole and urease. The enzymes coagulase and catalase properties failed to feature. Citrate utilization and oxidase were also negative.

Table 4; Biochemical Feature of*Haemophilus influenzae*

lactose	oxidase	catalase	coagulase	indole	urease	glucose	citrate	Bacteria
+	-	-	-	+	+	+	-	*Heamophilus influenzae*

DISCUSSION

An account of a significant difference in counts of*Haemophilusinfluenzae* withinPark, Market andHospital toilet seat bowls is quite usual and dissimilarto studies carried out by Matini et al. (2019). Matini et al. (2019) carried out a survey on public restroom and noted that public restrooms have too many counts of bacteria whereas this study failed to confirm. Matini et al. (2019) pointed out that the standard count for bacteria is 1000 CFU/ml. However, it was not stated if samples from seat swab were considered. Sampson et al. (2019), who worked on the toilet seats failed to identify counts for*Haemophilus influenzae*,but noted counts of heterotrophic bacteria at 2.7 x 10^5 CFU/ml. Heterotrophic bacteria counts noted by Sampson et al. (2019) werehowever, greaterthan counts of *Haemophilusinfluenzae* obtained in all sampling points in this study. The insignificant difference of *Haemophilus influenzae*counts recorded amongst the sampling point is also not in consonant with studies carried out by Archibald (2011), who in their study noted the highest bacteria population in public restroom is 1468 CFU/m^3 as against the lowest, of 480 CFU/m^3.The countsderived from seats of these public restroom although are not

detrimental as pointed out by Matini et al. (2019) who pointed detrimental effects fall between 10^4 and 10^5 CFU/g giving that the toilets are less intended to have greater loads of *Haemophilus influenzae*or any other bacteria as the sampling pointsunder study have high public usage.The advent of bacteria on toilet seat bowl and other surface is due to aerosolization during flushing of feces (biomatter) (Schreck et al., 2021). Aerosols desiccate and remain adrift in the air currents (Johnson et al., 2012) and later fall on surfaces. Thus, as a result of this, the identification of*Haemophilus influenzae*in this study is significant as the bacteria implication in respiratory tract infection can be fatal (Johnston & Apicella, 2009).Studies by Matini (2019) failed to identify out *Haemophilusinfluenzae in* restrooms, but *Escherichia coli, Staphylococcus aureus* and *Salmonella* spp.. Similarly, Archibald(2011),identified *Staphylococcus aureus, Micrococcus, Bacillus*spp,, *Klebsiella* spp.and*Cronobacter*isolated from restroom with exception of*Haemophilusinfluenzae* . Sampson et al. (2019) who worked on toilet seat did not identify *Haemophilus influenzae*but *Staphylococcus aureus, Bacillus, Klebsiella*spp.,and *Escherichia coli.*The greater prevalence of*Haemophilus influenzae*in hospital above the park and market restroom may have been pointed out by Best et al.(2012), where they pointed out that toilets in hospitals do not have lids, reason for high contamination of the toilet air environment immediately after flushing lidless toilet. However, studies of toilets in markets and park have not been specifically considered probably due to less provision of toilet facilities.

CONCLUSION

The study noted the presence of *Haemophilus influenzae*on toilet seats; this may serve as a source of disease transmission via inhalation. Thus, the presence of*Haemophilus influenzae*on

surface of toilet seat bowl resulted from aerosols generated during flushing. Hence, influenza infection (respiratory tract infection) may emerge.

RECOMMENDATION

Due to aerosilization after flushing feces, toilet seats should be closed before and after flushing at all times

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