OPEN ACCESS

African Journal of Microbiology Research

14 April 2018
ISSN 1996-0808
DOI: 10.5897/AJMR
www.academicjournals.org

ACADEMIC JOURNALS
expand your knowledge

ABOUT AJMR

The African Journal of Microbiology Research (AJMR) is published weekly (one volume per year) by Academic Journals.

The African Journal of Microbiology Research (AJMR) provides rapid publication (weekly) of articles in all areas of Microbiology such as: Environmental Microbiology, Clinical Microbiology, Immunology, Virology, Bacteriology, Phycology, Mycology and Parasitology, Protozoology, Microbial Ecology, Probiotics and Prebiotics, Molecular Microbiology, Biotechnology, Food Microbiology, Industrial Microbiology, Cell Physiology, Environmental Biotechnology, Genetics, Enzymology, Molecular and Cellular Biology, Plant Pathology, Entomology, Biomedical Sciences, Botany and Plant Sciences, Soil and Environmental Sciences, Zoology, Endocrinology, Toxicology. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles are peer-reviewed.

Contact Us

Editorial Office: ***ajmr@academicjournals.org***

Help Desk: ***helpdesk@academicjournals.org***

Website: ***http://www.academicjournals.org/journal/AJMR***

Submit manuscript online ***http://ms.academicjournals.me/***

Editors

Editors

African Journal of Microbiology Research

Table of Content: Volume 12 Number 14 14 April, 2018

ARTICLES

academicJournals

Vol. 12(14), pp. 327-332, 14 April, 2018
DOI: 10.5897/AJMR2017.8689
Article Number: 8CB50FD56788
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Evaluation of gene xpert assay and microscopic examination for detection of *Mycobacterium tuberculosis* in sputum at Port Harcourt, Nigeria

Confidence Kinikanwo Wachukwu[1*], Stella Paulinus Okwelle[2], Easter Godwin Nwokah[1] and Ollor Amba Ollor[1]

[1]Department of Medical Laboratory Science, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Rivers State, Nigeria.
[2]Department of Medical Microbiology, University of Port Harcourt Teaching Hospital, Choba, Rivers State, Nigeria.

Received 25 August, 2017; Accepted 14 December, 2017

The re-emergence and increase in the prevalence of tuberculosis cases and multidrug resistant strains is of public health concern. The conventional Acid Fast Bacilli detection tool is weak in the current disease trend especially in children, patients with human immunodeficiency virus (HIV) and low bacterial load. The aim of this research was to assess effectiveness of the Gene Xpert MTB/RIF assay and Microscopy in detectiing *Mycobacterium tuberculosis* (MTB) in sputum samples in Port Harcourt metropolis. A total of two hundred sputum samples were collected from *tuberculosis* (TB) suspected patients visiting the antiretroviral and directly observed short treatment clinics at Braithwaite Memorial Specialist Hospital, Port Harcourt and University of Port Harcourt Teaching Hospital, Choba. These samples were investigated to compare and evaluate the detection of *M. tuberculosis* using Gene Xpert MTB/RIF assay and microscopy. Results revealed that 20% (40/200) of the samples were positive for MTB. Ages 31 to 40 years had 55 samples with ten positive MTB while ages < 10 years had the least number of specimens and one MTB detected. Out of 200 samples analyzed, the percentage positive was 20% while 80% was negative for MTB. Ages < 10 years did not show any AFB positive smear whereas ages 31 to 40 years had 10 positive AFB smears. One hundred and four individuals were people living with HIV, ninety six were negative for HIV; fifteen individuals were positive for HIV as well as MTB (co-infection) and no individual that was co-infected had Rifampicin resistance. There is a significant difference in the detection of MTB across PLHIV and negative persons using AFB microscopy and Gene Xpert technique. It can be concluded that the gene xpert MTB/RIF assay is highly sensitive (98%) with a p-value of < 0.001 and specific in the detection of MTB and Rifampicin Resistance in sputum, and its use in health institutions should be encouraged to reduce exposure and spread of the disease as well correct treatment.

Key words: Gene xpert, tuberculosis, *Mycobacterium tuberculosis*/Rifampicin (MTB/RIF), sputum, microscopy.

INTRODUCTION

All over the world, Tuberculosis (TB) control agencies often adopt sputum smear microscopy alongside chest x-ray because of its economic value and simple laboratory procedure (Small and Pai, 2010). Using acid fast bacilli

(AFB) smear microscopy for the detection of tuberculosis in children, patients with low bacterial load and HIV is challenging hence the use of Xpert MTB/RIF which has great specificity and sensitivity for detecting Pulmonary *tuberculosis* (PTB) infection. An *in vitro* study when compared with approximately 10,000 colony forming units/ml with routine smear AFB microscopy showed a limit of detection of as few as 131 colony-forming units/ml(cfu/ml) of *Mycobacterium tuberculosis* (Dolin et al., 2010).

Performing drug susceptibility testing (DST) can be done from the growth of *M. tuberculosis* in culture, and this can take up to six weeks and needs high biological safety level laboratory which is expensive. Isoniazid and Rifampicin are two fundamental anti-TB drugs in use until recently when high resistance set in hence performing drug susceptibility testing became very important. In many countries, multi-drug-resistant tuberculosis (MDR-TB) is greatly increasing and its treatment is very challenging as it takes longer time with the use of several anti-bacterial agents that are expensive (Palomino et al., 2007).

The Xpert MTB/RIF assay technique was introduced in the United States by Cepheid Company, and was declared fit for use in December 2010 by the World Health Organization (2013) as a major tool for tuberculosis diagnosis worldwide. This declaration was made after about eighteen months of continuous effective field assessment of its use in tuberculosis multi drug resistance – TB and TB/HIV co-infection diagnosis (Van Rie et al*.,* 2010).

MDR-TB also known as Vank's Disease is defined as a form of TB infection caused by bacteria that are resistant to treatment with two of the most powerful first line anti-TB drugs such as isoniazid (INH) and Rifampicin (RIF). With the Xpert, MTB/RIF test MDR-TB diagnosis can be accomplished in fresh sputum samples and in prepared sediments within 2 to 3 h, microscopy cannot be used to detect tuberculosis that are resistant to drugs hence treatment is delayed. The quick detection of *M. tuberculosis* and Rifampicin resistance using Gene Xpert assay helps the medical personnel to make critical patient management decisions concerning treatment (Lalloo et al., 2006).

This test has similar sensitivity to culture, specifically detects *M. tuberculosis* as well as rifampicin resistance via the *rpoB* gene concurrently (WHO, 2013). It can be used in low-income setting to make patients access to early and accurate diagnosis easy, hence decreasing the death rate associated with delayed diagnosis and mistreatment (Fred, 2009).

The aim of this research work was to evaluate the performance of an automated system (Gene Xpert of *M.* MTB/RIF) and AFB smear microscopy for detection *tuberculosis* in sputum specimens. The specific objectives are to effectively diagnose *M. tuberculosis* in a significant number of individuals with negative AFB smears and to adequately diagnose patients that are resistant to TB drug Rifampicin.

MATERIALS AND METHODS

Study area

This study was carried out at the Tuberculosis Reference Laboratory (TBRL) University of Port Harcourt Teaching Hospital (UPTH) located along East West Road between Rumuosi and Choba town in Obi/Akpor Local Government Area of Rivers State, Nigeria. The Hospital shares a common boundary with the University of Port Harcourt, Abuja campus in Alakahia town of Rivers State. Patient using the directly observed treatment short course (Dots) and Antiretroviral (ARV) clinics in Braithwaite Memorial Specialist Hospital (BMSH) Port Harcourt and University of Port Harcourt Teaching Hospital (UPTH) were enrolled in the study. Those enrolled for this study were subjects between ages < 10 to 70 years.

Ethical approval

Ethical approval for this research work was granted by the Rivers State Health Research Ethics Committee while the informed consent of the patients used for this study was obtained before the commencement of the research.

Sample size

Two hundred sputum samples were collected from the individuals who enrolled, and analyzed according to Leslie Kish's formula, which was used to arrive at the sample size of 200:

$N = N^2 Pq/d^2$
Where N = sample size
Z = Z scores at 95% confidence level.
P = expected prevalence (in proportion of one: if 5% d = 0.05). For the level of confidence of 95% z value is 1.96 (Daniel 1999).

Inclusion criteria

These are individuals having cough for two weeks or more, retreatment cases of Pulmonary tuberculosis (PTB) within the last year or non-converting PTB case), persons that are in contact with individuals that have been recently treated of PTB, symptomatic presumptive TB cases with AFB negative results, to confirm MDR – TB, return after loss to follow up and people living with HIV (PLHIV) with symptomatic tuberculosis.

Patient's exclusion criteria

Individuals producing bloody sputum (haemoptysis) and sputum

*Corresponding author. E-mail: wachukwuck@yahoo.com, wachukwu.confidence@ust.edu.ng.

specimens with obvious food particles or other solid particulates were not accepted as these could interfere with the desired result. Also excluded from the study were patients who refused to participate or submit any sputum.

Specimen collection from adults

Sputum specimens were collected in duplicate; one part was used for making smear and the other for gene xpert. Patients were given two sterile wide mouthed containers each, they were told to rinse their mouth twice with water in the morning, unscrew the lid on the sputum collection container, inhale deeply and cough vigorously, expectorate the material into the two containers. They were advised to avoid spills or soiling the outside of the container, secure the lid on the collection device sample container labeled with name, age, sex, and date of collection according to directives previously reported in literature (Fred, 2009). Sputum specimens were held at 2 to 8°C whenever possible as they got blocks of pure water and safely put the samples between the blocks in sellable bags. The bags were three for each sputum specimen, with the form being placed on the third bag to avoid being soiled in case of any spillage. Both oral and structured questionnaire helped in accessing further details about individuals enrolled.

Specimen collection from children

In children, sputum specimen collection is quite difficult since they tend to swallow the sputum rather than expectorate it; therefore induced sputum specimens were collected especially from minor patients (age 5).

Methods of collection used

The methods used are nebulization techniques using hypotonic saline: Inhaling a nebulizer 3% sodium chloride mist for 5 to 15 min, and then encouraging them to cough and expectorate the sputum into a wide mouth container. Chest or abdomen massages technique: Chest percussion, vibration and active breathing were also used for sputum collection in minors (Grant, 2012).

Specimen processing

The sputum samples were collected by expectorating into sterile wide mouthed containers and liquefied with sputolysin (N-acetyl-L-cysteine), decontaminated with 4% NaOH alkali, which was neutralized with phosphate buffer and concentrated by centrifugation at 1500 to 2000 g for 20 min, specimens collected were processed for microscopic method and Gene Xpert MTB/RIF Assay, in a biosafety cabinet. In addition, appropriate safety gadgets were worn due to the hazardous nature of such samples.

Microscopic examination (Ziehl Neelsen staining technique)

The specimens were arranged after being numbered accordingly, slides were labeled on the frosted end with graphite pencil accordingly. The recommended size is 2 x 3 cm or 1 x 2 cm (Grant 2012). The smear was placed on the flat surface with smear facing upward. It was allowed to air dry for at least 1 h, and thereafter fixed by passing 3 times through a flame, ensuring that the smear face upward. This was done for all smeared samples. Fixed slides were arranged (in batch of 12) including controls on a processing rack, making sure that smear samples were separated from each other before staining. Slides were flooded with 10% strong carbol fuchsin, and heated to steam and allowed to stain for 5 min. Heating helps to melt the wax and opens up the mycolic cell wall to take in more stains, as the heat is removed they close up to retain the primary stain. Slides were rinsed in flowing tap water until no more colour runs off. Slides were thereafter flooded with 3% acid alcohol for 3 min to decolorize, and washed with running tap water to stop decolourization. Thereafter they were flooded with methylene blue (which serves as counter stain), for 1 min and rinsed with tap water, drained and dried. Slides were viewed using oil immersion, and under X100 objective lens.

Gene xpert MTB/RIF techniques

Sputum specimens with blood, obvious food particles or other solid particulates were not accepted as these could interfere with the desired result. Sputum samples on the original tube were used to mix with the sample reagent (which constitute of NaOH and Isopropanol which breaks down the mycolic cell wall of MTB while NaOH decontaminates the sputum samples) (that is, 4mls of sample reagent to 2 mls of sputum specimen, the ratio is 2:1 v/v) except in cases where the sputum is too mucoid, more of the sample reagent can be added as this will help to liquefy the mucoid sputum specimen easily. After the addition of the sample reagent, the mixture was shaken vigorously for 10 to 20 times, and incubated for 5 min at room temperature, and then shaken again for 10 to 20 times and incubated at room temperature for 10 minutes. Each xpert MTB/RIF cartridge was labeled with the sample identification (ID) on the sides of the cartridge, and using the sterile transfer pipette provided in the package of the cartridge packs. Two millilitres (2ml) of the liquefied sample was aspirated and transferred into the open port of the cartridge after opening the lid of the cartridge, this was done slowly to minimize the risk of aerosol formation. At this point hands were on stops while the machine automatically processes the samples with result displaced at the end of 1 h 90 min on the computer screen (Fred, 2009).

Statistical analysis

Statistical Package for Social Sciences (SPSS) Version 22 was used to check if there will be significant difference or not in the detection of MTB among the different age groups and PLHIV using Gene Xpert technique and AFB microscopy.

RESULTS

The numbers of sputum samples analyzed according to their age ranges are shown in Table 1. Most of the sputum samples 27.5% (55/200) were received or collected from patients ages 31 to 40; followed by ages 21 to 30 and 41 to 50 with 24.5 (49/200) and 21% (42/200), respectively. The least were from children under 10 with just 2% (4/200)". Of the 200 samples analyzed, 20% (40/200) were positive while 80% were negative for MTB, and this difference was statistically significant, $p = 0.4$ ($p>0.5$). Age range of 31 to 40 years old had the highest number of samples, with ten positive cases of *M. tuberculosis* (MTB). Ages < 10 years old had the least number of positive samples, with one MTB positive result. The number of AFB smear positive was 29 (14.5%) while 171 (85.5%) were smear negative. Ages < 10 years did not show any smear positive,

Table 1. Distribution of sputum samples according to age groups of patients

Age range (years)	No. of sputum	MTB Pos	MTB Neg	AFB Pos	AFB Neg
<10	4	1	3	0	4
11-20	18	4	14	1	17
21-30	49	12	37	10	39
31-40	55	10	45	10	45
41-50	42	3	39	2	40
51- 60	19	9	10	5	14
61 -70	13	1	12	1	12
Total	200	40 (20%)	160 (80%)	29 (14.5%)	171 (85.5%)

Table 2. Detection of TB and RIF determination by gene xpert technique.

MTB type	No. of sputum samples	Percentage
MTB Susceptible Positive (Detected) RIF	36	18
MTB Resistant Positive (Detected) RIF	4	2
Total	40	20

Table 3. Distribution of TB cases according to gender of patients.

Sex	No. of sputum samples	MTB Pos	AfB Pos	MTB Neg	AFB Neg
Female	116	19	15	97	101
Male	84	21	14	63	70
Total	200	40	29	160	171

whereas ages 21 – 30 and 31 – 40 years had 10 smear positive cases each. P = 0.007 (P<0.005), also shown in Table 1. Table 2 showed the rifampicin resistant cases; out of the 40 MTB detected sputum samples analyzed, 36 (18%) persons showed MTB positive while Rifampicin susceptible 4 (2%) were MTB positive and Rifampicin resistant. Table 3 shows the gender of MTB positive and negative as well as AFB smear positive and negative cases. Maximum number of the specimens was from females (58%) (116/200), and the rest from the males. HIV status of the various persons whose specimens were analyzed is shown in Table 4. Out of the 200 sputum samples one hundred and four persons were people living with HIV, while 96 persons were negative. Amongst 104 HIV positive persons, fifteen showed MTB detected which is a case of co-infection while eighty nine showed MTB negative.

DISCUSSION

Tuberculosis is one of the deadliest public health threats today. Annually, *M. tuberculosis* (MTB) causes about 8 to 9 million cases of infection as well as 1.5 million deaths (Issar, 2003). These numbers are on the rise globally, especially in Eastern Europe, Africa and the former Soviet Union. Part of the problem in treating TB is the appearance of drug resistant tuberculosis strains, including strains with multidrug resistance (MDR) and more recently strains with extensive drug resistance (XDR) which are more difficult to treat (Issar, 2003). In this part or area of the country (Rivers State, Nigeria) where this study was carried out, the rate of occurrence of all forms of TB cases in 2014 were 2,279 while in 2015 it increased to 2,369. This rise could have been due to several environmental factors such as poor housing and ventilation of the surroundings, overcrowding, and congregation in schools but exposure to ultraviolet light reduces it.

Then Xpert MTB/RIF assay, a non-laboratory based molecular assay according to Boehme et al. (2011) was designed specifically for use and quick asses to treatment in endemic disease settings, and it is the first diagnostic tests that have the potential to bring high sensitive nucleic acid amplification testing to peripheral sections of health system in this new generation.

Besides, the microscopy only takes about 24 to 48 h, while standard cultures can take 2 to 6 weeks for MTB to grow and conventional drugs resistance test can add 3 more weeks. Infection control decision is also reached

Table 4. HIV status of patients whose Samples were Analyzed.

HIV status	No of sputum samples	MTB Pos	AFB Pos	MTB Neg	AFB Neg
Positive	104	15	12	89	92
Negative	96	25	17	71	79
Total	200	40	29	160	171

quickly since diagnosis of infection is made within 2 h and patient attended to in just one contact (Boehme et al*.,* 2011).

This study revealed an overall infection of MTB to be 40 (20%). This number could have been due to the socioeconomic factors such as low-income people with large families, living in dense urban communities with inadequate housing conditions, people living in congregated institutions such as prisons, nursing homes for elderly people, social shelters, day nurseries and internally displaced persons camps (IDP). In addition as seen in this analysis, xpert MTB/RIF tool is highly sensitive as it was able to detect MTB positive in some persons with AFB smear negative, hence it is a highly recommended tool to be used for MTB detection than microscopy. This result was in agreement with the work by Boehme et al*.* (2011), with 94.4% sensitivity and 98.3% specificity for rifampicin resistance. Similarly, results from this study were also in agreement with what Small and Pai (2010) reported in relation or reference to a pooled sensitivity of 88 and 98% specificity. There is no significant difference in the detection of MTB among the different age groups in this study using gene xpert. That means using this technique, the detection rate among the groups will still be the same.

Boehme and Sutherland (2009) reported a sensitivity of 98% for patients classified as smear negative; culture positive and for culture positive samples xpert gave 100%. Scott et al*.* (2011) in their study found out that the xpert MTB/RIF non laboratory based molecular assay has potential to improve the diagnosis of tuberculosis (TB) especially HIV infected population through increased sensitivity, reduced turnaround time and immediate identification of Rifampicin (RIF) resistance.

In a clinical study conducted, the sensitivity of MTB/RIF test on just one sputum sample was 92.2% for culture positive TB and 98.2% for smear positive TB which also agrees with the present study. It was observed that the number of positive cases could have emanated due to high rate of HIV amongst the patients, for instance out of the 200 patients screened 104 (52%) were HIV positive which is significant enough to say that it is high. There is a significant difference in the detection of MTB across people living with HIV (PLHIV), and negative persons using AFB microscopy and Gene Xpert technique. This study also revealed 14% (15 cases) prevalence of MTB positive among HIV subjects, which was similar to the report of Scott et al. (2011) (86%) and Boehme et al*.* (2011), which showed high level of sensitivity in HIV MTB positive patients. No co-infected individual was found to be resistant to Rifampicin. The worrisome aspect of this study is that the high positivity rate of MTB was noticed amongst ages 21 to 30 years (30%) and age 31 to 40 years (25%) which is the age of the work force of most country and owing to this, productivity will be reduced which in turn will affect the economy of the country drastically if it is not checked.

Furthermore, the high MDR cases observed which if not properly managed, the dissemination rate may increase since these group of people attend the same churches, market and even join the same transport vehicles with others. Rifampicin resistant cases in this study was found to be 4 (2%), and treatment of this disease is very expensive since these patients have to be placed on second line drugs after further investigation's using culture and drug susceptibility testing (DST) to find out the actual drug to be used. For MDR cases, second line drugs are used, they include Amikacin, Kanamycin, Capreomycin and Etionamide. No indeterminate rate was observed (this could be attributed to the fact that the subject characteristics used in this study were well selected using inclusion and exclusion criteria) which corroborates with the work of Boehme et al. (2011) in which case the indeterminate rate was 2.4%.

There is a significant difference in the detection of AFB among the different age groups used in this study using AFB microscopy with P value < 0.05 which is significant; hence it is not a sensitive tool to be used. In addition, the number of AFB smear microscopy that showed positive in this study was twenty nine 29 (14.5%) as against forty (40) (20%) for the tuberculosis detected ones. This shows that with AFB microscopy alone most patients with positive cases could actually be missed if in every 40 positive cases with xpert MTB/RIF we are missing about eleven (11) as was shown with smear microscopy in this study the AFB sensitive rate which is low in this study also confirms or tallies with the work of Ritu and Vithal (2015) which gave AFB detection to be about 22 to 43%, though the High Ventilation Air Condition system (HVAC) at the facility was down at the time of carrying out this research we would have considered performing a concentration method for AFB to still make further comparison.

Conclusion

The study revealed that the new automated technique is

more sensitive, specific and more rapid in diagnosis of the disease in non HIV and HIV persons. This study has also shown that using this new technology will help to reduce exposure and transmission of the disease and also useful in detection of resistant strains.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Boehme C, Nicol MP, Nabeta P, Michael JS, Gotuzzo E, Tahirili R, Gler MT, Blakemore R, Worodria W, Gray C, Huang L, Caceres T, Albert H, Cobelens F, Cox H, Alland D, Perkins M. (2011). Feasibility, diagnostic accuracy, and effectiveness of decentralized use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a Multicentre Implementation Study. The Lanc. 377:1495-1505.

Boehme CC, Sutherland R (2009). Xpert, MTB/RIF Demonstrations for detection of TB and Rifampicin resistance. www.find diagnostic.org. document (Accessed 20/7/2015). 112-145.

Daniel WW (1999). Biostatistics, A foundation for analysis in the Health Sciences. (7th edition), New York: John Willey and Sons. 35-40.

Dolin F, Gerald L, Mandell E (2010). Principles and practice of infectious diseases. J. Infect. Dis. 250: 1124-1130.

Fred T (2009). Two-hour detection of MTB and resistance to rifampicin. *Invitro diagnostic medical device.* Diseases and Project-Tuberculosis-Xpert MTB/RIF Review A, 4300-7810 (Accessed 20/7/2015).

Grant LR (2012). Procedures for collection of induced sputum specimen from children. Clinic. Infect. Dis. Oxf. J. 54:140-145.

Issar S (2003). *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. Clinic. Microbiol. Rev. 16(3):463-496.

Lalloo G, Naidoo R, Ambaram A (2006). Recent advances in the medical and surgical treatment of multi-drug resistant tuberculosis. Curre. Opini. Pulmon. Medic. 12:179-185.

Palomino JC, Martin A, Portcels F (2007). Rapid drug resistance in *Mycobaterium tuberculosis*. Clinc. Microbio. Infect. 13:754-762.

Ritu S, Vithal PM (2015). Microscopy as a diagnostic tool in pulmonary tuberculosis. Intern. J. Mycobact. 4(1):1-5.

Scott LE, McCarthy K, Gous N, Nduna M, Van Rie A, Sanne I, Venter WF, Duse A, Stevens W (2011). Comparisons of Xpert MTB/RIG with other nucleic acid technologies for diagnosing pulmonary tuberculosis in a high HIV prevalence setting: A prospective study. J. Pub Medi. 8(7):1371-1379.

Small PM, Pai M (2010). Tuberculosis diagnosis – Time for a game change. New Engl. J. Med. 363:1070-1071.

Van Rie A, Page-Shipp L, Scott L, Sanne I. Stevens W (2010). Expert MTB/RIF for point-of-care diagnosis of TB in high HIV burden, resource-limited countries: hype or hope? Exp. Rev. Molec. Diagn. 10:937-946.

World Health Organization (2013). Updated WHO Recommendation for Diagnosis of Pulmonary TB, and Pediatric TB, Extra Pulmonary TB and Rifampicin resistance. www.who.int/tb/publications (Accessed 13/2/2016).

academicJournals

Vol. 12(14), pp. 333-337, 14 April, 2018
DOI: 10.5897/AJMR2016.8182
Article Number: 0F237BE56791
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Characterization of *p30* membrane protein gene of *Mycoplasma agalactiae* isolates by polymerase chain reaction and restriction endonuclease enzyme assay

Pranay K.[1,2*], Roy A.[1] and Aher T. K.[1]

[1]Department of Veterinary Microbiology, College of Veterinary Science and A. H. A.A.U., Anand-388001, Gujarat India.
[2]Department of Veterinary Science, CAFF, Fiji National University, Koronivia Campus, Nausori, #1544, Fiji Islands.

Received 24 June, 2016; Accepted 12 January, 2018

Seven hundred and eight (708) samples collected from the goats of Gujarat were screened for *Mycoplasma agalactiae* by culture and PCR using 16S rRNA based genus specific and species-specific primers. Amplification of the *p30* membrane protein gene was carried out using specific primers and the resultant amplicons were subjected to restriction enzyme analysis. The isolates yielded 715 bp and 360 bp products with genus-specific and species-specific primers, respectively and were identified as *M. agalactiae*. Amplification of the *p30* membrane protein gene yielded a 730 bp product. Restriction enzyme analysis of the 730 bp amplicon of *p30* gene with *Rsa*I and *MboII* yielded 2 fragments (654 bp and 74 bp) and 3 fragments (111 bp, 375 bp and 244 bp), respectively. Digestion with *Sau3A*I yielded two fragments (461 bp and 269 bp) while digestion with *Alu*I resulted in 3 fragments (342 bp, 328 bp and 60 bp). The results of the present study revealed the presence of polymorphism at the respective positions of *p30* membrane protein gene of *M. agalactiae* isolates examined by the restriction digestion. These polymorphisms can result into changes in pathogenesis and persistence inside the host and require further investigation of immunological outcome of these polymorphisms.

Key words: *Mycoplasma agalactiae*, polymerase chain reaction (PCR), restriction enzyme, *p30*.

INTRODUCTION

In small ruminants, *Mycoplasma agalactiae* is responsible for a syndrome known as contagious agalactiae of small ruminants (CASR) (Solsona et al., 1996), which is generally characterized by mastitis, arthritis and keratoconjunctivitis. It causes reduction and suppression of milk production (De Garnica et al., 2013) and occasionally results into abortion and death (Madanat et al., 2001). For pathogenesis and clinical manifestations of mycoplasmal infections, adhesion to host cells and immune evasion are the major prerequisite for colonization. Different strategies used for the successful persistence of *M. agalactiae* inside host include a

*Corresponding author. E-mail: drpranaykumar@gmail.com.

constantly changing surface structure and the capacity of some lipoproteins to induce the expression of up- and down modulating cytokines (Razin et al., 1998). Few constantly expressed surface proteins have also been described in *M. agalactiae* which includes *p30*, *p48* and *p80* as immunogens and other proteins belonging to the variable surface membrane proteins family (*Vpma*) (Cacciotto et al., 2010). *Vpma* phase variation has been reported to have importance for survival and persistent infection of *M*. *agalactiae* in host (Chopra-Dewasthaly et al., 2017).

The *M*. *agalactiae* variable gene (*avg*) system is a cluster of four genes that encode a family of surface lipoproteins characterized by high-frequency phase and size variations (Flitman-Tene et al., 2000). Stability and variation of these membrane proteins have implications for immunogenicity and pathogenicity. Among the immunodominant membrane proteins, expression of *p30* was detected consistently from several strains of *M. agalactiae* revealing its importance for serological analysis (Fleury et al., 2001). Membrane proteins p80 (Kashoo et al., 2011) and *p40* (Fleury et al., 2002) were also reported to have serological importance in the *M. agalactiae* infections and their importance as candidate proteins for diagnosis.

Several studies have targeted these membrane protein genes for identification of the organisms (Macun et al., 2010). Among the various molecular techniques, restriction analysis is an important technique of genetic characterization and to study the variation among the strains of *Mycoplasma* circulating in the field. In the present study, *p30* membrane protein gene was targeted to study the polymorphism among the isolates obtained from the samples collected from goats of Gujarat, which has significant number of goat and sheep population including several well-known milch and dual purpose breeds of India.

MATERIALS AND METHODS

Collection and processing of samples for cultural isolation

Ear (169), nasal (158) and ocular (28) swabs as well as lung tissues (94) and milk (259) samples were collected aseptically from healthy as well as sick animals and processed for isolation of *Mycoplasma*. After collection, each sample was placed directly in 2 ml of MBHS-L broth (Modified Balanced Hank's Salt Solution Liquid Media) and kept at 37°C for 1 h. After incubation for 1 h, 200 µl of each MBHS-L broth containing sample was transferred to 2 ml of fresh MBHS-L broth after filtration (with 0.45 µm filter). The fresh MBHS-L broths containing filtered inoculum were incubated at 37°C for 10-15 days. The broths were examined daily for sign of growth (floccular material) and positive cultures were further purified. The samples showing growth in broth were inoculated on solid media (Modified Balanced Hank's Salt Solution Agar Media, MBHS-A) and incubated anaerobically for 10 days at 37ºC under humid conditions in 5-10% carbon dioxide tension to obtain optimum growth (Carmichael et al., 1972).

Examination of the isolated colonies and biochemical characterization of isolates

The suspected colonies of *Mycoplasma* were examined morphologically under microscope (4X) after staining them with different stains viz. Dienes', Giemsa and Acridine orange stain. Biochemical tests as described by Erno and Stipkovits (1973) such as catabolism of glucose, hydrolysis of arginine, phosphatase activity, tetrazolium reduction, serum digestion, digitonin sensitivity test (Freundt, 1973) and film and spot formation were carried out to determine the biochemical activity of the suspected *Mycoplasma* isolates.

Confirmation of *Mycoplasma* isolates by polymerase chain reaction (PCR)

For preparation of the template DNA, 2 ml of broth culture of each isolate was centrifuged at 12000 rpm in a micro-centifuge at 4°C for 25 min. The pellets were washed in 500 µl of PBS twice; pellets were resuspended in 100 µl of nuclease-free water and boiled for 10 min. After boiling, the suspension was snap-chilled at -20°C for 5 min. After chilling, cell debris was removed by centrifugation and 3 µl of the supernatant was used as a DNA template in PCR after quantitation and quality assessment of DNA using Nano-drop spectrophotometer. For identification of genus and species of *Mycoplasma*, the PCR was carried out in a final reaction volume of 25 µl using 200 µl capacity PCR tube containing 3 µl of DNA template, 1 µl of each primer (10 pmole/µl), 12.5 µl of 2X PCR Master-mix (Fermentas) and 7.5 µl of DNase-RNase free water.

The isolates were confirmed as *Mycoplasma* sp. using genus specific forward, GPO-1 (5'-ACTCCTACGGGAGGCAGCAGTA-3') and reverse, MGSO (5'-TGCACCATCTGTCACTCTGTTAACCTC-3') primers amplifying 715 bp fragment of 16S rRNA of *Mycoplasma* sp. (Kuppeveld et al., 1992) after PCR. After initial denaturation at 94°C for 2 min, amplification was carried out for 30 cycles (consisting of denaturation at 94°C for 45 s, annealing at 55°C for 1 min and extension at 72°C for 1 min) with final extension at 72°C for 5 min.

For confirmation of species as *M. agalactiae*, PCR was performed using *M. agalactia*e specific forward, Maga (5'-CCTTTTAGATTGGGATAGCGGATG-3') and reverse Maga (5'-CCGTCAAGGTAGCGTCATTTCCTAC-3') primers for an expected amplification product of 360 bp fragment of the 16S rRNA gene (Chávez-González et al., 1995). After initial denaturation at 95°C for 5 min, amplification was carried out for 40 cycles (consisting of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 68°C for 1 min) with final extension at 70°C for 10 min.

To detect the targeted amplification, 5 µl of PCR product from each tube was mixed with 1 µl of 6X gel loading buffer and electrophoresed on 1.5% agarose gel at a constant 80V for 30 min in 0.5X TBE buffer along with 100 bp DNA Ladder (GeneRuler-Fermentas). It was then stained with ethidium bromide (1% solution at the rate of 5 µl/100 ml), the size of the product was visualized under UV light and documented by the gel documentation system (SynGene, Gene genius bioImaging System, UK).

Amplification of *p30* gene and restriction enzyme analysis

The membrane protein gene *p30* of four representative isolates of *M. agalactiae* was amplified using the specific forward, P30(F) (5'-CAGGGGGATGAACATTTATG-3') and reverse, P30(R) (5'-TTACCTCCATCTTTTTCAAC-3') primers (Fleury et al., 2001) in a final reaction volume of 25 µl (containing 3 µl of DNA template, 1 µl of each primer (10 pmole/µl), 12.5 µl of 2X PCR master-mix

Figure 1. Agarose gel electrophoresis of PCR product of *p30* gene of *M. agalactiae*. Lanes 1 - 4: Positive representative samples. L: 100 bp DNA molecular weight marker

(Fermentas) and 7.5 µl of DNase-RNase free water) for an expected amplicon of 730 bp. After initial denaturation for 2 min at 94°C, thermal cycling was carried out for 35 cycles (denaturation at 94°C for 30 s, annealing at 52°C for 45 s and extension for 1 min at 68°C) with final extension for 5 min at 72°C.

The amplified PCR products of membrane protein gene were further processed and characterized by RE analysis. Four different REs, *viz. Rsa*I, *Mbo*II, *Sau3A*I and *Alu*I were selected from restriction map created using the sequences of *p30* membrane protein gene available in GenBank at *http://www.ncbi.nlm.nih.gov/Genbank/index.html* and NEBcutter V2.0 software available online at *http://tools.neb.com/NEBcutter2/index.php.* A (30 µl) reaction mixture (containing 10 µl of PCR product, 1 µl of RE (10 U/µl), 2 µl of 10X restriction buffer and 17 µl of nuclease free water) was prepared and incubated in a water bath overnight according to the conditions specified by the manufacturer (Fermentas). After restriction digestion, an aliquot (10 µl) of each digested PCR product was mixed with 2 µl of gel loading buffer and electrophoresed along with 100bp DNA molecular weight marker on 2% agarose gel containing ethidium bromide (1% @ 5 µl/100 ml) by submarine gel electrophoresis apparatus at constant voltage of 60V for 45 min in 0.5X TBE buffer. After completion of electrophoresis, the gel was examined on UV transilluminator to observe the various fragments and photographed by gel documentation system (SynGene, Gene Genius BioImaging System,UK).

RESULTS

All the 13 isolates were identified on the basis of colony morphology and biochemical characters. In broth, floccular deposits were observed whereas on Modified Balanced Hank's Salt Solution Agar **(**MBHS-A) medium, the typical fried egg appearance of colonies of Mycoplasmas were observed after staining. Biochemically, the isolates were sensitive to digitonin and positive for tetrazolium reduction, phosphatase production and film and spot test. All the isolates were

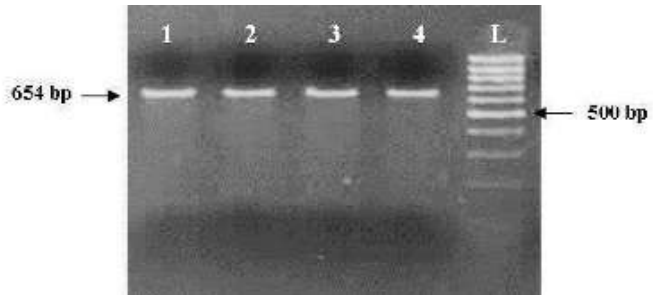

Figure 2. *Rsa*I generated RE pattern of PCR product of *p30* gene of *M. agalactiae* showing larger restriction fragment (654 bp) and unresolved smaller fragment (76 bp). Lane 1 - 4: Digested PCR product of representative isolates of *M. agalactiae.* L: 100 bp DNA molecular weight marker.

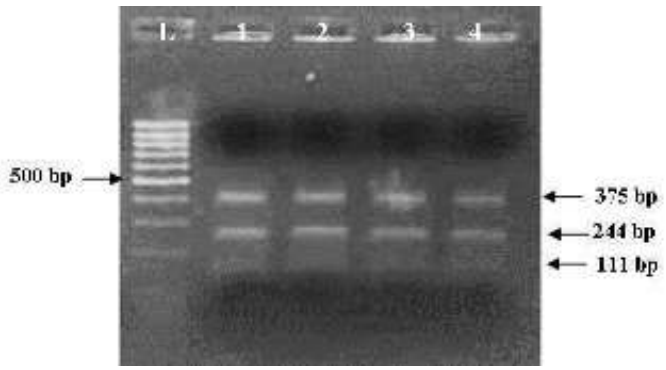

Figure 3. *Mbo*II generated RE pattern of PCR product of *p30* gene of *M. agalactiae* showing 3 restriction fragments (375 bp, 244 bp and 111 bp). Lane 1 - 4: Digested PCR product of representative isolates of *M. agalactiae.* L: 100 bp DNA molecular weight marker

negative for glucose metabolism, arginine hydrolysis and serum liquefaction. The isolates were further confirmed as *M. agalactiae* by PCR using genus and species specific primers which yielded specific amplification product of 715 bp and 360 bp respectively. The *p30* gene with amplicon size of 730 bp was detected in all the representative *M. agalactiae* isolates analyzed (Figure 1).

Digestion of the 730 bp product of the *p30* gene with *Rsa*I yielded a single large fragment of 654 bp along with the smaller 76 bp fragment which could not be resolved because of its smaller size (Figure 2). Analysis of the products with *Mbo*II revealed the presence of three fragments of 111 bp, 375 bp and 244 bp in size (Figure 3). Digestion of the amplified products with the *Sau3A*I yielded two fragments of 461 bp and 269 bp (Figure 4). Digestion of the amplified product with *Alu*I yielded two fragments resolved into bands of 342 bp and 328 bp, whereas one smaller fragment of 60 bp could not be resolved (Figure 5).

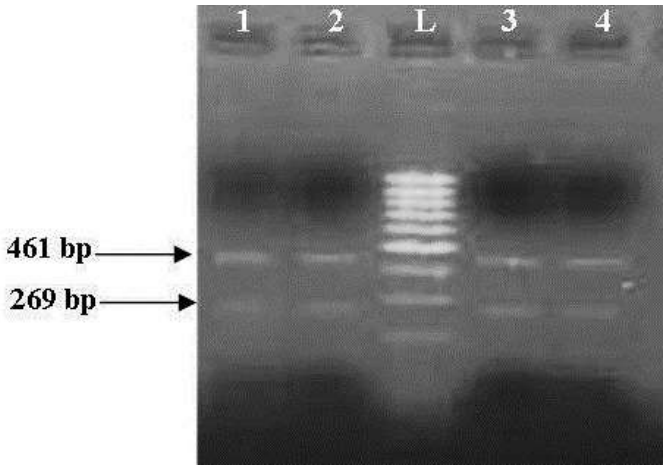

Figure 4. *Sau3A*I generated RE pattern of PCR product of *p30* gene of *M. agalactiae* showing 2 restriction fragments (461 bp and 269 bp). Lane 1 - 4: Digested PCR product of representative isolates of *M. agalactiae.* L: 100 bp DNA molecular weight marker.

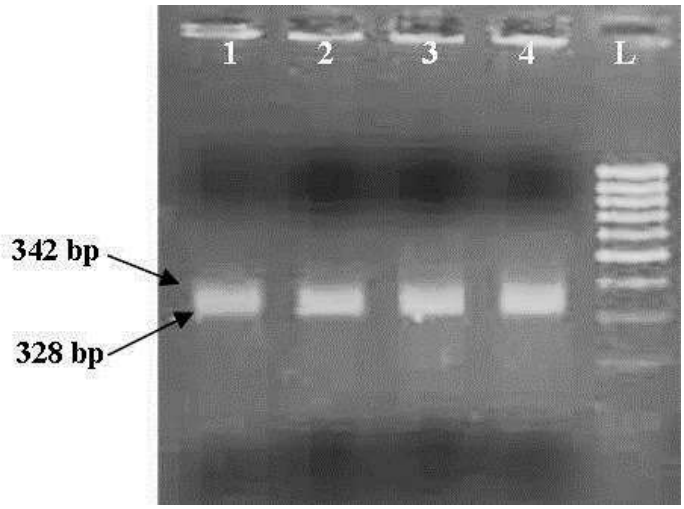

Figure 5. *Alu*I generated RE pattern of PCR product of *p30* gene of *M. agalactiae* showing 2 restriction fragments (342 bp and 328 bp, unseparated) with unresolved smaller fragment (60 bp). Lane 1 - 4: Digested PCR product of representative isolates of *M. agalactiae.* L: 100 bp DNA molecular weight marker.

DISCUSSION

Restriction patterns produced by *RsaI* and *MboII* were in accordance with the expected restriction of *M. agalactiae p30* gene revealing the presence of one and two restriction sites respectively. Variations in the expected pattern were observed in case of digestion with *Sau3A*I and *Alu*I. Digestion with *Sau3A*I yielded only two fragments of 461 bp and 269 bp in contrast to the three expected fragments of 461 bp, 105 bp and 164 bp according to the restriction map showing the absence of one restriction site. Digestion of *p30* amplicons with *Alu*I revealed the absence of one restriction site resulting into a larger fragment of 328 bp in contrast to the expected two fragments of 282 bp and 46 bp along with other fragments of 60 bp and 342 bp fragments. Thus, the samples revealed the presence of only two restriction sites in contrast to the expected three restriction sites as per the expected restriction map with *Alu*I.

Several studies have been undertaken on the genetic characterization of the isolates by restriction digestion of various genomic segments of *M. agalactiae* including membrane protein gene. Glew et al. (2002) studied the polymorphism of the *vpma* locus of *M. agalactiae* by RE analysis using *Ase*I, *Alw*I and *Hind*III. An identical banding pattern was observed for both *Ase*I and *Alw*I whereas different patterns were observed with *Hind*III. In a similar study, Sung et al. (2006) analyzed the nested PCR products of various mycoplasmal strains by restriction enzyme digestion with *Sau*3AI enzyme to further identify and differentiate between the *Mycoplasma* species including *M. agalactiae*. Macun et al. (2010) targeted the 81 kDa membrane protein gene for the detection of *M. agalactiae* from cases of contagious agalactiae by PCR. In agreement with the present study, Kashoo et al. (2011) carried out the RE analysis of PCR products of *p80* gene of *M. agalactiae* using *Rsa*I and *Xho*I restriction enzymes to confirm the identity of the amplified products which produced the expected fragments of 146 and 868 bp with *Rsa*I enzyme, while 176 and 838 bp fragments were obtained with *Xho*I enzyme on 2.5% agarose gel electrophoresis.

Conclusion

The present study reveals the presence of polymorphism using PCR-RE analysis among the strains of *M. agalactiae* prevailing in the field in Gujarat region of India. This western province has significant population of sheep and goat which are affected by several disease condition and many disease outbreaks have been reported in them. Screening of those flocks is important to understand the cause of morbidity and mortality. Molecular tools are very important for rapid screening of those flocks as well as for characterization of the associated pathogens. The present study also shows the importance of molecular tools in the study of genomic variation among the field strains of these microorganisms causing covert and overt manifestations utilizing various mechanisms to evade host immune response. Many studies globally have shown the importance of RE analysis in the study of polymorphism in various genomic segments of field strains of *M. agalactiae* and development of detection system for different species.

These findings further underline the need to study the immunological implications of these variations occurring in the membrane proteins of these mycoplasmal pathogens which can ultimately affect their pathogenesis and persistence in the host.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are thankful to the Professor and Head, Department of Veterinary Microbiology and Dean, College of Veterinary Science and Animal Husbandry, Anand (Gujarat), for providing the facilities to pursue this work.

REFERENCES

Cacciotto C, Addis MF, Pagnozzi D, Chessa B, Coradduzza E, Carcangiu L, Uzzau S, Alberti A, Pittau M (2010). The liposoluble proteome of *Mycoplasma agalactiae*: an insight into the minimal protein complement of a bacterial membrane. BMC Microbiol. 10:225.

Carmichael LE, Sullivan ND, Hoasfall N (1972). Isolation, propagation and characterization studies of an ovine *Mycoplasma* responsible for proliferative interstitial pneumonia. Cornell. Vet. 62:654-679.

Chávez-González Y, Bascunana CR, Bolske JG, Mattson JB, Molina CF, Johansson KE (1995). *In vitro* amplification of the 16S rRNA genes from *Mycoplasma bovis* and *Mycoplasma agalactiae* by PCR. Vet. Microbiol. 47:183-190.

Chopra-Dewasthaly R, Spergser J, Zimmermann M, Citti C, Jechlinger W, Rosengarten R (2017). *Vpma* phase variation is important for survival and persistence of *Mycoplasma agalactiae* in the immunocompetent host. PLoS Pathog. 13(9):e1006656.

de Garnica ML, Rosales RS, Gonzalo C, Santos JA, Nicholas RAJ (2013). Isolation, molecular characterization and antimicrobial susceptibilities of isolates of *Mycoplasma agalactiae* from bulk tank milk in an endemic area of Spain. J. Appl. Microbiol. 114:1575-1581.

Erno H, Stipkovits L (1973). Bovine Mycoplasmas: cultural and biochemical studies. Acta. Vet. Scand. 14:436-449.

Fleury B, Bergonier D, Berthelot X, Peterhans E, Frey J, Vilei EM (2002). Characterization of P40, a cytadhesin of *Mycoplasma agalactiae*. Infect. Immun. 70(10):5612-21.

Fleury B, Bergonier D, Berthelot X, Schlatter Y, Frey J, Vilei EM (2001). Characterization and analysis of a stable serotype-associated membrane protein (*p30*) of *Mycoplasma agalactiae*. J. Clin. Microbiol. 39(8):2814-2822.

Flitman-Tene R, Levisohn S, Lysnyansky I, Rapoport E, Yogev D (2000). A chromosomal region of *Mycoplasma agalactiae* containing *vsp*-related genes undergoes *in vivo* rearrangement in naturally infected animals. FEMS Microbiol. Lett. 191:205-212.

Freundt EA, Ernø H, Black FT, Krogsgaard-Jensen A, Rosendal S (1973). Evaluation of reference reagents for *Mycoplasmas*. Ann. N. Y. Acad. Sci. 225:161-171.

Glew MD, Marenda M, Rosengarten R, Citti C (2002). Surface diversity in *Mycoplasma agalactiae* is driven by Site-Specific DNA inversions within the *vpma* Multigene Locus. J. Bact. 184(21):5987-5998.

Kashoo ZA, Singh VP, Rana R, Sankar M, Gazalli H, Susan S (2011). Molecular characterization of P80 gene of Indian *Mycoplasma agalactiae* isolates. Ind. J. Anim. Sci. Sci. 81(5):3-7.

Macun HC, Ocal N, Karahan M, Yagci BB, Kalender H, Kalim, R (2010). Endemic contagious agalactiae in sheep and goats: Clinical evaluation, Treatment and Vaccination. J. Anim. Vet. Adv. 9(14):1918-1924.

Madanat A, Zendulkova D, Pospisil Z (2001). Contagious agalactia of sheep and goats: A review. Acta Vet. Brno. 70:403-412.

Razin S, Yogev D, Naot Y (1998). Molecular biology and pathogenicity of mycoplasmas. Microbiol. Mol. Biol. Rev. 62:1094-1156.

Solsona M, Lambert M, Poumarat F (1996). Genomic, protein homogeneity and antigenic variability of *Mycoplasma agalactiae*. Vet. Microbiol. 50(1-2):45-58.

Sung H, Kang SH, Bae YJ, Hong JT, Chung YB, Lee C, Song S (2006). PCR based detection of *Mycoplasma* species. J. Microbiol. 44(1):42-49.

Van-Kuppeveld FJ, Van-der-logt JT, Angulo AF, Van-Zoest MJ, Quint WG, Niesters HG, Galama JM, Melchers WJ (1992). Genus and species specific identification of Mycoplasmas by 16S rRNA amplification. Appl Environ Microbiol. 58:2606-2615.

Related Journals: