ORIGINAL RESEARCH ARTICLE

"EVALUATION OF BRAIN SAMPLE COLLECTION THROUGH FORAMEN MAGNUM VIS-A-VIS CONVENTIONAL SKULL OPEN METHODS FOR DIAGNOSIS OF RABIES IN SUSPECTED ANIMALS"

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Abstract

Laboratory based diagnosis of rabies in animals is the pre requisite for institution of suitable control strategies. However, this depends on the submission of quality brain samples to the laboratory. Presently, collection and submission of brain samples from field for the laboratory confirmation of suspected cases of rabies is a major constraint in rabies surveillance and in turn evolving control strategies. This can be attributed to the conventional, laborious method of brain sample collection based on the skull cap open method. There is urgent need for a easy method that is user friendly, rapid and risk free for collection of brain samples at the field level and also to know the accuracy in use of Lateral Flow Assay (LFA) for rapid diagnosis of rabies in animals. In the present study, 51 post-mortem brain samples (cerebellum and brain stem) were collected from rabies suspected animals. Brain samples were collected by employing Foramen Magnum approach using simple instruments *viz.,* Artificial Insemination (AI) sheath, which is easily available in all Veterinary Dispensaries and also collected from Conventional skull cap open method. The Lateral flow Assay (LFA), Direct Fluorescent Antibody Assay (DFA) and direct rapid immunohistochemistry (dRIT) tests were employed and comparative evaluation of all three tests was done on samples collected from both the methods. Out of 51 samples tested, 41 were positive and 10 negative for rabies viral inclusions by LFA, DFA and dRIT tests. There was 100 per cent correlation between the performance of LFA, DFA and dRIT with respect to the positivity and negativity of brain samples for rabies viral antigen / inclusions. Furthermore, the results of all the three tests were the same when the samples were collected by both Skull cap open and Foramen Magnum approaches. Since there is no variation in the test results with respect to the type of tests or method of brain sample collection, the user friendly, rapid and risk free collection of brain samples through Foramen Magnum at the field level can be successfully employed. The LFA can be used as a primary and Rapid diagnostic tool in field level conditions prior to DFA based confirmation of the same in the laboratory. This encourages collection and submission of more number of brain samples from field for the laboratory confirmation of rabies and inturn effective animal rabies surveillance.

Key words: Rabies, Brain sample collection, Foramen Magnum approach, Artificial Insemination sheath,

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Introduction

Rabies is an invariably fatal, zoonotic disease of importance and endemic in many parts of the world. It is caused by neurotropic, enveloped RNA virus belonging to the family *Rhabdoviridae.* Two clinical forms of rabies, namely, furious and dumb forms are observed in animals. The clinical signs such as behavioral changes including aggression, drooping jaw and tongue, salivation, restlessness and paralytic signs exhibited by the affected animals are suggestive of rabies, but need confirmation. Apart from rabies, such nervous signs are seen in other neurological diseases due to infectious, metabolic and nutritional causes. Thus, an accurate diagnosis of animal rabies is possible only through laboratory examination of infected materials collected from the diseased animals.

The most widely used test for postmortem rabies diagnosis is fluorescent antibody test / Direct fluorescent antibody Assay (FAT/DFA) which is recommended by both World Health Organization (WHO) and World Organization for Animal Health (OIE) .

Currently, the DFA is considered to be the gold standard for diagnosis of rabies. However, the development and evaluation of a rapid immuno histochemical test namely Direct Rapid Immunohistochemical Test (dRIT) at the CDC, Atlanta is a mile stone. The dRIT procedure takes one hour and fifteen minutes and has the advantage of applicability under field conditions as expensive fluorescence microscope, deep freezer and incubator are not required unlike DFA. The test has been evaluated in field under variable conditions of preservation. This increases the suitability of this test for use in field conditions in developing countries, where cold storage facilities may not be available. Furthermore, the dRIT has undergone extensive evaluation in several countries and 100% correlation was found with DFA. Yet, another recently described method for the detection of rabies virus antigen from postmortem samples is the rapid immunodiagnostic test (RIDT), a useful method for rabies diagnosis without the need for laboratory equipments. This immuno-chromatographic lateral flow strip test is a one-step test that facilitates low-cost, rapid identification of viral antigen. Such lateral flow assay can be used as a rapid screening test in animals.

All though, an array of laboratory tests are now available for diagnosis of Rabies, the collection of appropriate samples from field cases and sending them to the laboratory for diagnosis is associated with a number of practical issues at the field level. Opening the skull cap is laborious and thought to be rhisky when practiced outside laboratories and therefore, the entire carcass or head of the suspected animal is often dispatched to the laboratory. This can be particularly difficult under the field conditions prevailing in remote areas and tropical countries, where the field worker has not only to find suitable containers to pack bulky specimens but also have means to keep the specimen cool while it is transported to the laboratory in addition to the availability of vehicles and manpower for the transportation of carcass to the laboratory. Such logistical problems of transportation of complete carcass / or decapitated head of the carcas to the laboratory can be overcome by submitting only the brain sample. This is possible provided the filed veterinarian is confidant of post mortem collection of brain sample.

Opening of the skull cap for collection of brain specimens for rabies diagnosis is a relatively laborious, time consuming and delicate procedure that should be performed by a well trained person. It also requires special precautions to avoid accidental exposure to the virus through wounds or by aerosol. In view of this, internal brain sampling without autopsy by the introduction of a disposable plastic pipette / juice drinking straw via the occipital foramen (Barrat and Halek, 1986) or via the retro-orbital route (Montano Hirose *et al*., 1991) appeared to be particularly rapid and safe in field conditions. However, juice drinking straw being soft, might collapse and may not be user friendly and unsuitable for sample collection.

For large livestock, such as cattle and horses, shipping of the entire head to a diagnostic laboratory / center poses special problems. For these animals, portions of the brainstem and cerebellum can be removed by the veterinary clinician through the Foramen magnum following decapitation at the occipito axial juncture (Debbie and Trimarchi, 1992).

The classical method to collect the brain samples from rabid suspected animals can be done by opening the skull cap which is time-consuming operation (Zerai Woldehiwet, 2005 and Shankar, 2009). Furthermore, brain

sample meant for polymerase chain reaction (PCR) require typical methods that preclude contamination from other specimens (Kadam *et al.,* 2011).

In view of these limitations, field workers involved in rabies diagnostic work would benefit from a simple, user friendly and rapid postmortem technique by which appropriate brain samples can be collected, tested by employing the LFA and dispatched to laboratory for further confirmatory diagnosis of rabies.

To overcome these limitations, an attempt was made in this study

1. To standardize the methodology of brain sample collection from suspected cases of rabid animals (with special reference to dogs) through Foramen magnum approach.
2. To evaluate immuno-diagnostic tests using brain samples collected from conventional skull open and foramen magnum approach.

METHODOLOGY :

Brain samples (cerebellum and brain stem) were collected from rabies suspected animals presented to Department of Veterinary Pathology, Veterinary College, KVAFSU, Bengaluru- 560024 for rabies diagnosis during October 2017 to June 2018. For assessing the efficacy/usefulness of different sample collection approaches and to establish their feasibility in the field level, brain samples were collected both by Foramen magnum approach and conventional skull cap opening method.

During the post mortem examination, brain stem was collected from the Foramen magnum approach and cerebellum was collected from skull open method in the same animal. In all, 51 samples were collected which included dogs (n=46), cattle (n=3), cat (n=1) and horse (n=1).

Procedure of brain sample collection through Foramen magnum approach

Initially, the carcass was kept on Post mortem table in the lateral recumbency and the head was flexed ventrally. A deep incision was made just behind nuchal crest of occipital bone severing the Skin, Cutaneous fascia Cervicoscutularis muscle, Splenius, Brachiocephalicus muscles and the insertion point of nuchal ligament to expose occipito-atlantal joint . The joint was then dislocated using a sharp disposable scalpel blade. This exposed the Foramen magnum which seats some parts of Pons, Medulla oblongata and major parts of brainstem. An artificial insemination (AI) sheath cut to the required size depending on the species of animals was connected to a disposable syringe was then inserted deeply into the Foramen magnum and the tissue from brain stem was aspirated into the AI sheath. The brain sample was collected in sample storage container and submitted to the Laboratory in cold chain for further processing.

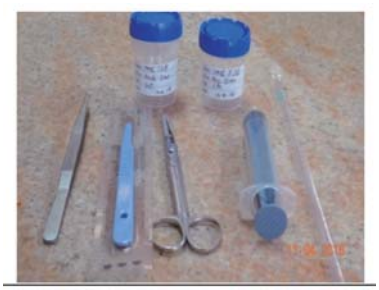

Fig.1.1: Equipments for brain sample collection through Foramen Magnum method

Fig.1.2: Identification of Occipito-Atlanto joint behind the nuchal crest.

Fig.1.3: Severing of skin & muscles to expose Occipito-Atlanto joint.

Fig 1.4: Insertion of AI sheath connected to a disposable syringe deeply into the Foramen magnum and the tissue from brain stem was aspirated.

Procedure of Brain sample collection through Skull cap open approach

This is the conventional method, by which the brain samples were collected from the same animals from which brain sample were already collected through Foramen magnum. In brief, the skin and temporal muscles were retracted to expose the bones of the skull (Fig 1.5). The head was then secured firmly and cuts were made through the skull, either with an oscillating saw or a chisel. The brain sample collected in sample storage container and sent to Laboratory for further process

Fig.1.4: Instruments for brain sample collection through Open Skull method

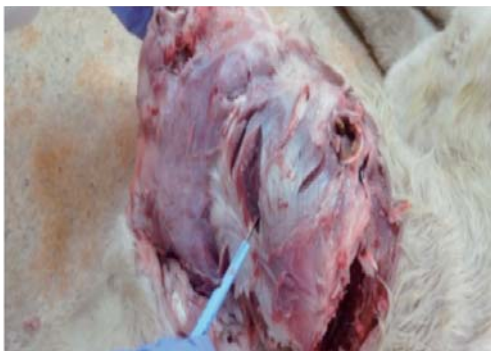

Fig 1.5: Skin and Temporal muscles were retracted to expose the bones of the skull

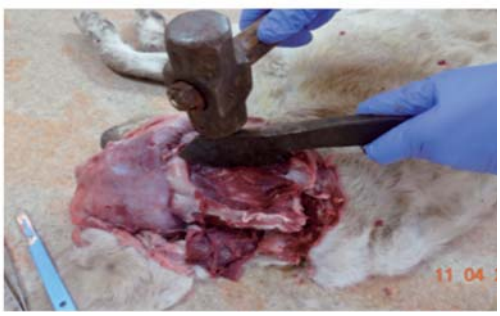

Fig 1.6: Bones of skull were cut using chisel and hammer to expose the brain

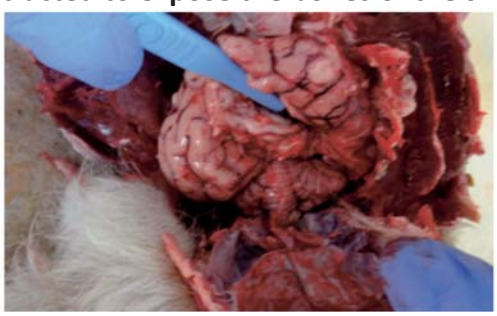

Fig 1.7 : Bones of skull cut using chisel and hammer to expose the brain and collecting cerebellum

Rabies suspected brain samples (n=51) from various domesticated species (*viz.,* dog, cat, cattle and horse) were collected from different geographical locations following the above mentioned procedures. (Table1.1). Specimens thus collected were transferred to leak proof rigid sample containers with cold chain and were shifted to the KVAFSU-CVA Rabies Diagnostic Laboratory, Dept of Microbiology, Veterinary college, KVAFSU, Hebbal, Bengaluru at the earliest.

Table 1.1: Region-wise and species-wise details of sample collection

Area → / Species	Bangalore North	Bangalore South	Bangalore East	Bangalore West	Total
Dogs	18	09	10	9	46
Cattle	1	-	-	2	3
Cats	-	-	1	-	1
Horses	-	1	-	-	1
TOTAL	19	10	11	11	51

In all, 51 brain samples collected by both the Foramen magnum and Skull cap open methods were subjected to DFA and dRIT as per Nithin Prabhu *et al.* (2018) and the LFA as per the instructions of the manufacturer of the kit (Bionote[R], South Korea). The presence or absence of typical apple green fluorescence of aggregated nucleocapsids in background with red colored brain tissue in slides processed for DFA and brick red colored viral inclusions in the background of blue stained nerve tissue in case of slides processed for dRIT were used as a criterion for declaring positive and negative samples. In case of LFA, presence of red colored lines at both C (Control) and T (Test) positions in the cassette was considered positive where as presence of single line at C position was considered negative for virl antigen in the brain suspension.

Results

In the present study, a comparative evaluation of different diagnostic techniques in rabies *viz.,* DFA, dRIT and LFA using brain samples (n=51) collected from conventional skull cap open and Foramen magnum approach was carried out and Laboratory techniques intended for diagnosis of rabies were preferably conducted on fresh brain tissue samples.

Out of total 51 brain samples examined, 41 samples were found positive for rabies antigen by DFA, dRIT and LFA (Table 1.2; Fig1.8, Fig1.9 and Fig 1.10). Remaining 10 samples were found Negative for rabies antigen in all the above three tests.

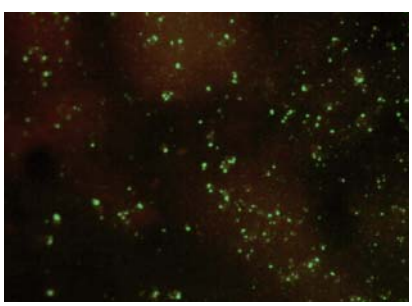

Fig 1.8

Brain impression of rabid dog (SAMPLE No.634) collected using Foramen magnum method showing apple green fluorescence by DFA

DFA X 400

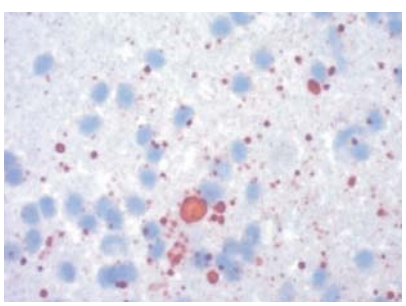

Fig 1.9

Brain impression of rabid dog (SAMPLE No.634) collected using Foramen magnum method showing brick red coloured viral inclusions of varying size scattred thoughout.

dRIT X 200

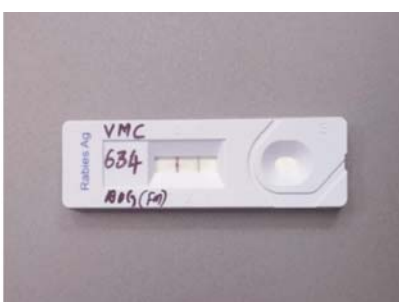

Fig 1.10

Lateral flow assay using the Antigen Rapid Rabies Ag Test Kit of BIONOTE of brain sample (SAMPLE No.634) collected by Foramen magnum approach showing red colored line at position ' T' indicating positivity for rabies antigen.

Table1.2: Comparative evaluation of different tests and approaches employed for brain material collection for diagnosis of rabies in animals.

Method & Organs	Species	No of samples	Results in different tests						Total	
			DFA		dRIT		LFA			
			+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Skull open approach										
a. Brain stem	Dog	46	39	7	39	7	39	7	**39**	**7**
	Cattle	3	2	1	2	1	2	1	**2**	**1**
	Cat	1	-	1	-	1	-	1	**-**	**1**
	Horse	1	-	1	-	1	-	1	**-**	**1**
b. Cerebellum	Dog	46	39	7	39	7	39	7	**39**	**7**
	Cattle	1	2	1	2	1	2	1	**2**	**1**
	Cat	1	-	1	-	1	-	1	**-**	**1**
	Horse	1	-	1	-	1	-	1	**-**	**1**
Foramen magnum approach										
a. Brain stem	Dog	46	39	7	39	7	39	7	**39**	**7**
	Cattle	3	2	1	2	1	2	1	**2**	**1**
	Cat	1	-	1	-	1	-	1	**-**	**1**
	Horse	1	-	1	-	1	-	1	**-**	**1**
b. Cerebellum	Dog	46	39	7	39	7	39	7	**39**	**7**
	Cattle	3	2	1	2	1	2	1	**2**	**1**
	Cat	1	-	1	-	1	-	1	**-**	**1**
	Horse	1	-	1	-	1	-	1	**-**	**1**
Total		51	41	10	41	10	41	10	**41**	**10**

Comparative evaluation of diagnostic test:

In all, 51 brain samples were collected with majority of the samples from dogs (n=46), and the rest from other domesticated species *viz.,* cattle (n=3), cat (n=1) and horse (n=1) .All the brain samples collected by both Foramen magnum and Skull cap open methods were screened for rabies viral inclusions by employing DFA, dRIT and rabies Antigens by LFA. In all, 41 positive cases (n=41) were found positive for rabies viral inclusions by DFA and all these samples were also found positive by dRIT and LFA. The remaining 10 samples were found to be negative by all the three tests.The percent positivity of positive and negative samples for rabies was same in all the 3 tests evaluated.. In the present study, tests were performed on the fresh brain samples collected by both Foramen magnum and Skull cap open approaches. The sensitivity and Specificity of both the approaches were 100% and correlated with all the three tests.

Discussion

In the present study, 51 rabies suspected brain samples were collected from different species of animals including dogs (n=46) and the rest were from other domesticated species *viz.,* cattle (n=3), cat (n=1) and horse (n=1). They were subjected to DFA, dRIT and LFA for diagnosis of rabies and also to compare sensitivity and specificity. It was observed that 41 out of 51 cases examined were found to be positive from samples collected from both skull open method and foramen magnum approach. The AI sheaths used for collecting brain samples through Foramen magnum approach were found suitable. It was found to be very easy to collect using AI sheath than plastic pipettes

or Fruit juice straws since AI sheaths are available in all Veterinary Dispensaries / Institutions and are strong unlike juice drinking straws.

Comparative evaluation of Brain Sample collection- by Foramen Magnum and Occipital Foramen approach

Comparative evaluation of various diagnostic techniques in 51 rabies suspected cases revealed the percentage of positivity of 100.00 for DFA, dRIT and LFA. The results of the present study indicated that all the three tests were equally efficient in detection of rabies positive cases. The sensitivity and the specificity of dRIT and LFA were carried out in relation to DFA which showed 100 percent positivity with the formula proposed by Thrusfield (2007). DFA is regarded as a Gold standard test. In the present study, 51 Brain samples were collected by both approaches *viz.,* FM and SO methods. They were screened for DFA, dRIT and LFA results were 100 per cent correlating between the tests and between the methods of collection also. Zerai Woldehiwet, (2005) and Shankar (2009) indicated that classical method to collect the brain samples from rabid suspected animals is by opening the skull which is a time consuming and risky operation. Furthermore, WHO, TRS 982 report and Kadam *et al*. (2011) research says that brain sample meant for polymerase chain reaction (PCR) require typical methods that preclude contamination from other specimens.Barrat and Halek, (1986) opined that, opening of the skull for collection of brain specimens for rabies diagnosis is a relatively long and delicate procedure that should be performed by well trained technicians. It also requires special precautions to avoid accidental exposure to the virus through wounds or by aerosol. So Internal brain sampling without autopsy by the introduction of a disposable plastic pipette via the occipital foramen appeared to be particularly rapid and safe in field conditions. Debbie and Trimarchi, (1992) found that for large livestock, such as cattle and horses, shipping of the entire head to a diagnostic laboratory / center poses special problems. For these animals, portions of the brainstem and cerebellum can be removed by the veterinary clinician through the foramen magnum following decapitation at the occipito axial juncture.Bingham and Maria van der. (2002) opined that sample collection was more reliable when taken from the occipital foramen than through other routes because this route is certain to include parts of the brain stem. They also opined that sampling of the brain has the advantage in that it is not always necessary to open the cranium which is a difficult, time consuming and probably a hazardous procedure when undertaken in the field. It also helps in saving the transport cost of sending the brain tissue sample (usually brain stem portion) to laboratory rather than sending the whole brain.

According to Bingham *et al*. (2002) and Iamamoto *et al*. (2011) both occipital foramen Route (OFR) and retro-orbital routes (ROR) were suitable for brain specimen collection and also they are equally sensitive and specific when compared with that of classical method. They stated that both the techniques were equally sensitive and could be used in field conditions when a laboratory structure was not available for sampling. Field veterinarians could also adopt these techniques, as it is very simple, rapid and safe. They concluded that both ROR and OFR for collection of brain sampling was comparable to the classical method of brain sampling and useful for further epidemiological surveillance of rabies. Further, Silva *et al*. (2013) indicated that this type of collection reduces the cross contamination, easy to transport brain samples from field to lab and minimizes the chances of accidental exposure to the virus. The rapid collection procedures described are also suitable for total RNA extraction in rabies diagnosis by the polymerase chain reaction because only disposable materials are used like AI sheath and drinking straws. According to Iamamoto *et al*.(2011) sampling brains from large mammals using this technique might prove beneficial to the extent that it will protect the collectors accidentally cutting themselves with possible rabies-contaminated saw blades. They indicated that, freezing of the samples for transport can be avoided, as these samples are ideal for preservation and transportation in 50% glycerol–saline solution, when freezing temperatures are not available. Also, they mentioned that collection of brain samples from wildlife species with the use of plastic pipettes stands out for many reasons: it can be applied in a short time, causes no damage to the skull. Above all, safe, easy to collect and thus improves rabies surveillance. In the present study, Artificial Insemination (AI) sheaths have been used for collecting brain samples through Foramen magnum. It was found to be very easy to collect using AI sheath than plastic pipettes or fruit juice straws since AI sheaths are easily available in field conditions.

Comparative evaluation of Direct Fluorescent Antibody Assay (DFA), Direct Rapid Immunohistochemistry (dRIT) and Lateral Flow Assay (LFA) In the present study, various diagnostic techniques *viz.,* DFA, dRIT and LFA well correlated in detection of positive and negative cases, indicating that all the three diagnostic tests were equally efficient in diagnosis of rabies in animals. Earlier, Genovese and Andral, (1978); Anjaria and Jhala, (1985); Hamir and Moser,(1994); Chandrashekhara, (2013) and Mundas (2013) have done the comparative studies on the efficacy of dRIT and DFA tests, results indicated that both were shown to be 100 per cent sensitive and specific. Kang *et al*.(2007) evaluated a Rapid Immunodiagnostic test kit for rabies virus detection using 51 clinical samples and 4 isolates of rabies virus. They found that the rapid immunodiagnostic test had a sensitivity of 91.70 per cent and specificity of 100 per cent. In their study, among the 51 clinical samples 44 were brain specimens and 7 were salivary samples. It is known that, rabies virus is secreted intermittently in saliva & other secretions, which might be the reason for getting less sensitivity by Rapid Immunodiagnostic test kit in those samples. Later, Mundas, (2013) has screened 114 brain samples suspected for rabies by sellar's, FAT, dRIT and RT-PCR for confirmatory diagnosis of rabies. Of these 77 samples were found to be positive by both FAT & dRIT. There was 100% correlation between both DFA and dRIT. In yet another study, Nithin, (2014) screened 200 brain samples for RABV by DFA, dRIT and RT-PCR. Both DFA and dRIT could detect RABV antigens in 129 samples whereas RT-PCR could detect the presence of RABV genome in an additional 35 samples. In all, 129 of the 200 samples were positive by both the tests, indicating 100 per cent correlation between them. Recently, Tajunnisa, (2017) collected brain samples from rabies suspected animals involving different host species from seven Indian states and compared the effect of acetone and formalin based fixation of brain impressions in the diagnosis of rabies in animals by DFA and LFA. In all, she screened 200 samples by DFA and LFA and found Sensitivity and specificity of LFA to be 99.2 and 98.5 per cent respectively. Minor difference in Sensitivity & Specificity of LFA was attributed to decomposition due to poor preservation of brain samples and long time transport from other states. The aforementioned studies are in conformity with our findings.

Conclusions

From the observation of the present study, it could be concluded that,

1. The brain samples collected by both the approaches *viz.,* FM and SO methods showed 100% sensitivity and specificity by DFA, dRIT and LFA. Further, FM approach can be effectively used for brain tissue collection and avoid sending entire carcass for laboratory examination.
2. All the three diagnostic techniques *viz.,* DFA, dRIT and LFA detected equal numbers of positive and negative cases suspected for rabies.indicating that they are equally efficient and reliable in diagnosis of rabies in animals.
3. dRIT and LFA could be used for the screening of the suspected samples at field level as diagnosis could be made by using limited resource and time and their accuracy is comparable to that of DFA.

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